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Espectro de mutações somáticas em tumores com origem em locais diferentes do intestino grosso em doentes com predisposição hereditária para cancro colo-rectal

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Spectrum of somatic mutations in tumors arising in different large bowel locations in patients with hereditary predisposition to colorectal cancer

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SUMMARY

Lynch syndrome is an autosomal dominant disorder characterized by a high incidence of early-onset colorectal cancer (CRC) and extracolonic tumors of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary, and hepatobiliary tract, accounting for up to 4% of all CRC. This syndrome is commonly caused by a genetic defect affecting one of the four mismatch repair genes *MLH1*, *MSH2*, *MSH6* and *PMS2*. The germline mutational spectrum of Lynch syndrome is highly heterogeneous, but specific mutations are observed at high frequencies in well-defined populations or ethnic groups, because of founder and/or recurrent effects. The identification of founder and/or recurrent mutations facilitates the molecular diagnosis of Lynch syndrome by making cost-effective mutational analysis of specific gene regions before full screening of all MMR genes.

More than 95% of the tumors arising in carriers of MMR gene mutations present microsatellite instability (MSI), due to MMR deficiency. Through the MSI pathway, colorectal cancer progression is accelerated by a rapid mutation accumulation in coding repetitive sequences of target genes with growth-related, apoptosis and DNA repair functions. We have previously reported in sporadic MSI CRC that the target genes in MMR deficient tumors in distal colon and rectum differ from those occurring elsewhere in the colon. This study aimed to ascertain the relative contribution of the underlying molecular defect driving carcinogenesis, on one hand, and the site of tumor origin, on the other, for the pattern of acquired genetic changes in CRC from patients with Lynch syndrome.

To determine if two frequent mutations in Lynch syndrome families, one of them novel and the other described worldwide, are founder and/or recurrent mutations, we performed haplotype analyses in Portuguese Lynch syndrome families. In order to characterize the spectrum of somatic mutations in CRC from patients with Lynch syndrome, we analyzed a series of CRC for microsatellite instability in genes predicted to be selective target genes of MSI and known to be involved in several

pathways of colorectal carcinogenesis, especially the TGFβ superfamily and WNT pathways.

Our results indicate that the novel exonic rearrangement *MLH1* c.1896+279_oLRRFIP2:c.1750-678del and the point mutation *MSH2* c.388_389del mutation are two frequent Portuguese founder mutations, the latter being a recurrent mutation worldwide. The high frequency of these two founder mutations in our Lynch syndrome families indicates that screening for these two mutations as a first step may increase the cost-effectiveness of genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal.

Regarding the somatic mutational spectrum of Lynch syndrome CRC, we detected a high frequency of mutations in genes belonging to the TGFβ superfamily signaling pathways. We also observed that CRC from Lynch syndrome patients with germline mutations in *MLH1* or *MSH2* present a significantly higher frequency of target gene mutations when compared to patients with germline *MSH6* mutations. A higher mean frequency of mutations in other genes with microsatellite sequences was also observed in CRC with somatic mutations in *MSH3* and/or *MSH6* when compared to CRC without somatic mutations in these two MMR genes. Finally, mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* genes were significantly more frequent in CRC with origin in distal colon in Lynch syndrome patients. To conclude, the pattern of somatic genetic changes differs in colorectal carcinomas depending on the large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different pathways even if driven by generalized MSI.

RESUMO

A síndrome de Lynch é uma doença autossómica dominante caracterizada por uma elevada incidência de carcinoma colo-rectal (CCR) e ocorrência de outras neoplasias, nomeadamente no endométrio, estômago, intestino delgado, uréter, bacinete, ovário e tracto hepatobiliar, representando até cerca de 4% de todos os CCR. Alterações nos genes MMR estão na origem desta síndrome, nomeadamente nos genes *MLH1*, *MSH2*, *MSH6* e *PMS2*. O espectro mutacional da síndrome de Lynch é muito heterogéneo, mas mutações específicas são observadas com elevadas frequências em populações bem definidas ou grupos étnicos devido a efeitos fundadores e/ou recorrentes. A identificação de mutações fundadoras e/ou recorrentes facilita o diagnóstico molecular da síndrome de Lynch porque direcciona a pesquisa de mutações para regiões específicas antes da análise total dos genes MMR.

Mais de 95% dos tumores em portadores de mutações nos genes MMR apresentam instabilidade de microssatélites (IMS). Na via de carcinogénese da IMS a progressão do CCR é acelerada pela rápida acumulação de mutações em sequências repetitivas de genes alvo com funções relacionadas com o crescimento celular, apoptose e reparação de DNA. O nosso grupo reportou num trabalho anterior em CCR esporádico com IMS que os genes alvo da via da IMS são diferentes entre tumores com origem no cólon distal e proximal. Este estudo teve como objectivo avaliar a contribuição relativa da alteração molecular germinativa, por um lado, e o local de origem do tumor, pelo outro, no padrão de alterações genéticas em CCR de doentes com síndrome de Lynch.

De forma a determinar se duas mutações muito frequentes em famílias portuguesas com síndrome de Lynch, sendo que uma delas é nova e a outra está descrita em vários países, são mutações fundadoras e/ou recorrentes, efectuámos estudos de haplótipos. Com o intuito de caracterizar o espectro de mutações somáticas em CCR de doentes com síndrome de Lynch, avaliamos a IMS em potenciais e supostos genes alvo relevantes nas vias envolvidas na carcinogénese colo-rectal, particularmente as vias do TGFβ e WNT.

Os nossos resultados indicam que o rearranjo exónico *MLH1* c.1896+279_oLRRFIP2:c.1750-678del e a mutação pontual *MSH2* c.388_389del são duas mutações fundadoras Portuguesas, sendo que a última também é recorrente a nível mundial. A elevada frequência destas duas mutações justifica que a sua pesquisa seja efectuada antes da análise completa dos genes MMR, o que poderá traduzir-se num aumento da relação custo-benefício no diagnóstico genético de síndrome de Lynch de famílias portuguesas, especialmente nas famílias originárias do norte de Portugal.

Relativamente ao espectro mutacional dos CCR de doentes com síndrome de Lynch, foi detectada uma elevada frequência de mutações nos genes da via de sinalização do TGFβ. Também observámos que CRC de doentes com mutações germinativas nos genes *MLH1* ou *MSH2* apresentavam maior frequência média de mutações nos genes alvo quando comparado com os doentes com mutação germinativa no gene *MSH6,* sendo esta diferença significativa. Adicionalmente, também foi detetada uma maior frequência média de mutações nos genes alvo em CRC que apresentavam mutações somáticas nos genes *MSH3* e/ou *MSH6* quando comparado com os casos sem alterações somáticas nestes genes. Finalmente, mutações nas sequências microssatélites (A)7 do gene *BMPR2* e (A)8 do gene *MSH3* foram significativamente mais frequentes em CCR com origem no cólon distal. Em conclusão, o padrão de mutações somáticas em CCR é diferente consoante o local de origem do tumor e entre CCR de doentes com síndrome de Lynch e CCR esporádico com IMS, sugerindo que a carcinogénese colo-rectal associada à IMS pode ocorrer por diferentes vias mesmo quando é impulsionada por IMS generalizada.

LIST OF PUBLICATIONS

PAPER I

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PAPER II

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PAPER III

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LIST OF ABBREVIATIONS

5 – FU	5- Fluorouracil
ACVR2A	*Activin A receptor, type IIA*
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of variance
APC	*Adenomatous Polyposis Coli*
ASR	Age-standardized rate
ATP	Adenosine triphosphate
AXIN	Axis inhibition protein
B2M	*Beta-2-microglobulin*
BAT	Big polyadenine tract
BAX	*Bcl-2 associated X protein*
BCL10	*B-Cell CLL/Lymphoma 10*
BMP	Bone morphogenetic protein
BMPR1A	*Bone morphogenetic protein receptor, type IA*
BMPR2	*Bone morphogenetic protein receptor, type II*
BRAF	*V-RAF Murine Sarcoma Viral Oncogene Homolog B1*
CACNA1G	*C*alcium channel, voltage-dependent, T type, alpha 1G
CASP5	*Caspase 5*
CRC	Colorectal cancer
CIN	*Cromossomal instability pathway*
CIMP	CpG island methylator phenotype
CTNNB1	*Catenin (cadherin-associated protein) beta 1*
E2F4	*E2F transcription factor 4, p107/p130-binding*
EGFR	*Epidermal growth factor receptor*
EPCAM	*Epithelial cell adhesion molecule*
FAP	Familial Adenomatous Polyposis
FOLFOX	Folinic acid/Fluorouracil/Oxaliplatin
FoSTeS	Fork stalling and template switching
GDP	Guanosine diphosphate

GTP	Guanosine triphosphate
HNPCC	*Hereditary Non-Polyposis Colorectal Cancer*
IGF2	*Insulin Growth Factor 2*
IGF2R	*Insulin Growth Factor 2 Receptor*
IHC	Immunohistochemistry
INSIGHT	International Society for Gastrointestinal Hereditary Tumors
KRAS	*Kristen Rat Sarcoma Viral Oncogene homolog*
LOH	*Loss of heterozygosity*
LRRFIP2	*Leucine rich repeat (in FLII) interacting protein 2*
MAP	MUTYH-associated polyposis
MAPK	*Mitogen-activated protein kinases*
MGMT	*O-6-methylguanine-DNA methyltransferase*
MLH1	*MutL E. Coli homolog of 1*
MLH3	*MutL E. Coli homolog of 3*
MMR	Mismatch repair genes
MSH2	*MutS E. Coli homolog of 2*
MSH3	*MutS E. Coli homolog of 3*
MSH6	*MutS E. Coli homolog of 6*
MSI	Microsatellite instability
MSI-H	Microsatellite instability High
MSI-L	Microsatellite instability Low
MSS	Microsatellite stable
NAHR	Nonallelic homologous recombination
NCCN	National Comprehensive Cancer Network
NEUROG1	*Neurogenin 1*
NHEJ	Non-homologous end-joining
NRAS	*Neuroblastoma RAS Viral Oncogene Homolog*
PMS1	*Postmeiotic segregation increased S. Cerevisiae 1*
PMS2	*Postmeiotic segregation increased S. Cerevisiae 2*
PRDM2	*PR domain containing 2*
PTEN	*Phosphatase and tensin homolog*

RUNX3	*Runt-related transcription factor 3*
SMAD 2	*Mothers Against Decapentaplegic Drosophila, Homolog of 2*
SMAD 4	*Mothers Against Decapentaplegic Drosophila, Homolog of 4*
SOCS1	Suppressor of cytokine signaling 1
TCF7L2	*Transcription factor 7-like 2 (T-cell specific, HMG-box)*
TGF-β	*Transforming Growth Factor β*
TGFBR2	*Transforming Growth Factor β Receptor type II*
TNM	Tumor, Node, Metastasis
TP53	*Tumour Protein 53*
UICC	International Union Against Cancer
VEGF	*Vascular endothelial growth factor*
WNT	Wingless-Type MMTV Integration Site Family

INTRODUCTION

1 - Anatomy and embryology of the large bowel

The large bowel constitutes a tube of variable diameter with approximately 120 to 150 cm in length and can be divided in cecum, ascending colon, transverse, descending colon sigmoid and rectum (Figure 1). The cecum is a capacious saclike segment localized in the proximal colon, with an average diameter of 7.5 cm and length of 10 cm. The ascending colon, approximately 15 to 20 cm in length, runs upward toward the liver on the right side. The transverse colon is approximately 30 to 60 cm in length and is located between the hepatic and splenic flexures. The descending colon extends downward from the splenic flexure for approximately 20 to 25 cm, followed by the sigmoid colon that varies in length from 15 to 50 cm. The rectum, which is 10 to 15 cm in length, ends in the proximal border of the anal sphincter complex (Petras et al, 2008; Fry et al, 2012). Usually, the different segments of the large bowel are classified as proximal or distal depending if they are located proximally or distally to the splenic flexure. The segments from cecum to transverse colon are considered proximal colon and from descending colon to rectum as distal colon (Petras et al, 2008). Regarding the anatomic blood supply, the proximal colon is supplied by the superior mesenteric artery and distal colon by the inferior mesenteric artery. The parasympathetic supply to the proximal colon comes from the right vagus nerve, whereas distal colon receives sympathetic supply from the preganglionic lumbar splanchnics of L1 to L3 and the lower rectum receives from the postganglionic sacral splanchnics of S2 to S4 (Petras et al, 2008; Fry et al, 2012).

The primitive gut tube has its origin in the endodermal roof of the yolk sac and at the beginning of the third week of development, being divided into three regions: the foregut, the midgut and the hindgut. Foregut-derived structures end at the second portion of the duodenum, the midgut gives origin from the duodenal ampulla to the distal transverse colon, and the hindgut from the distal third of the transverse colon to the rectum. The primitive distal rectum presents a specialized area denominated

cloaca, composed of endoderm and ectoderm derived tissues (Junqueira and Zago, 1997; Fry et al, 2012).

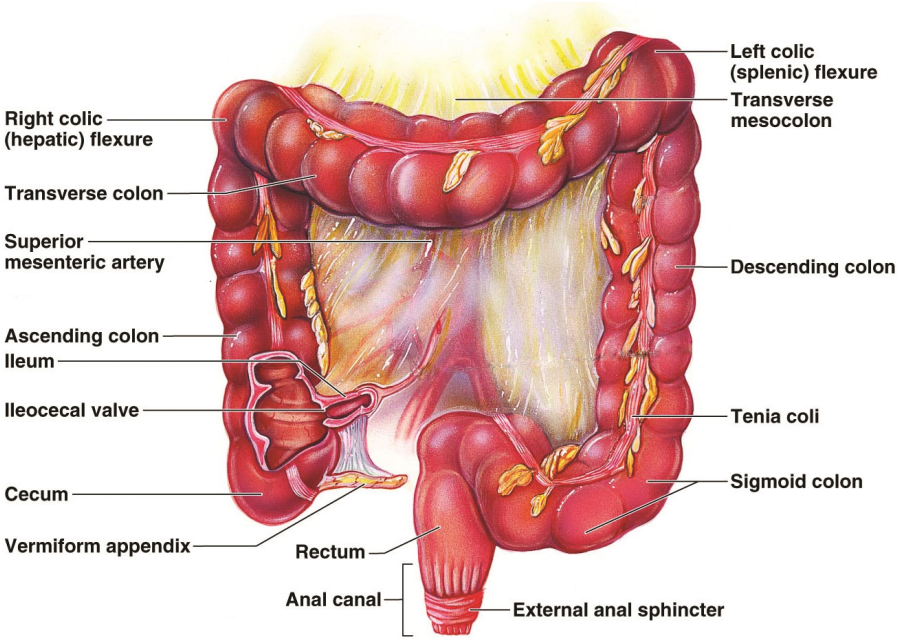

Figure 1 - Anatomy of the large bowel (adapted from Marieb et al, 2008).

The major functions of the large bowel are nutrient and water absorption and fecal storage, occurring differentially in the various segments of the large bowel. Nutrient and water absorption take place mostly in the cecum and decreases towards the rectum, and the sigmoid and the rectum serves as a fecal reservoir. Structural and physiological differences of the segments that constitute the large bowel, as well as the mucosal capillary density and width and the bacterial flora, explain this functional heterogeneity (Bresalier, 2010; Fry et al, 2012).

2 - Colorectal cancer incidence and mortality rates

The frequency of colorectal cancer (CRC) varies widely among different populations, presenting the highest incidence rates in Europe, North America, and Oceania and the lowest in Africa and South America (Ferlay et al, 2013). In 2012, CRC was the fourth most incident malignancy, preceded by breast, prostate and lung cancers, accounting for 9.7% (1 360 602 cases) of all new cancer cases diagnosed in

the world, with an estimated age-standardized rate (ASR) incidence of 17.2/100 000 (Ferlay et al, 2013). CRC accounted for 8.5% (693 881 deaths) of all deaths from cancer, being the fourth most common, preceded by lung, liver and stomach cancers, with an estimated ASR mortality of 8.4/100 000. Regarding gender, CRC was the third most common cancer in men and the second in women, with an estimated ASR incidence of 20.6/100 000 and 14.3/100 000, respectively (Ferlay et al, 2013). CRC represents the fourth most common cause of death from cancer in men and the third in women, with an estimated ASR mortality of 10.0/100 000 and 6.9/100 000, respectively (Ferlay et al, 2013).

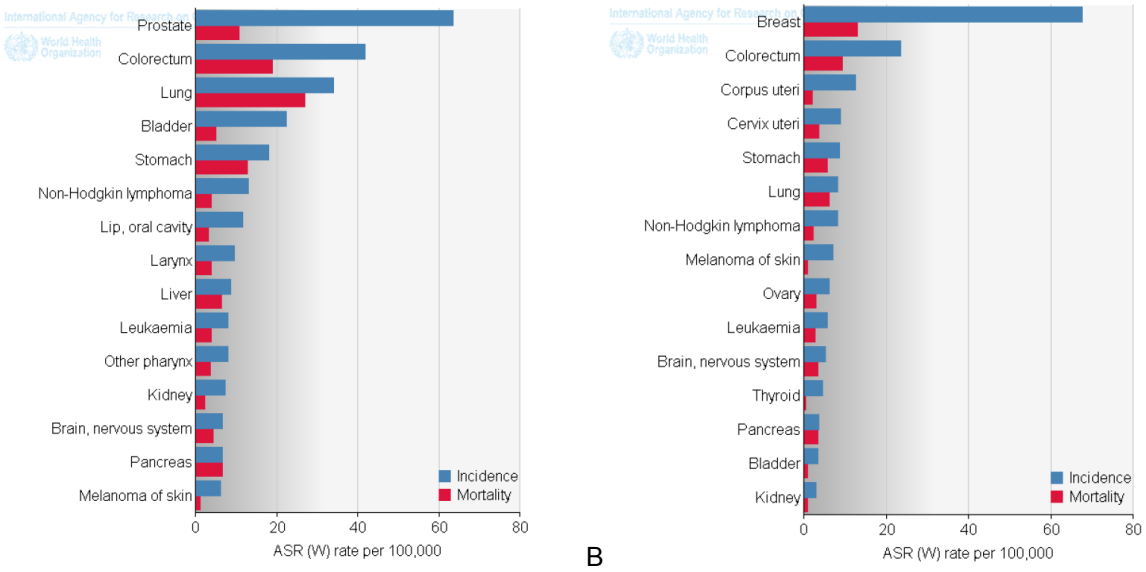

Figure 2 - Incidence and mortality age standardized rates (ASR) in men (A) and women (B) in Portugal in 2012 (Ferlay et al, 2013).

In Portugal, in 2012, CRC accounted for 14.5% (7129 cases) of all new cancer cases, being the third most incident malignancy with an estimated ASR incidence of 31.7/100 000 preceded by breast and prostate cancer (Ferlay et al, 2013). CRC was the second most common cause of death from cancer (3797 deaths) preceded only by lung cancer, accounting for 15.7% of all deaths from cancer with an estimated ASR mortality of 13.6/100 000. Considering gender, CRC was the second most common cancer in men and women, preceded only by prostate and breast cancer, with an estimated ASR incidence of 41.8/100 000 and 23.6/100 000, respectively

(Ferlay et al, 2013). Regarding mortality CRC, was the second most common cause of death in men and women with an estimated ASR mortality of 19.0/100 000 and 9.4/100 000, preceded only by lung and breast cancer, respectively (Figure 2) (Ferlay et al, 2013).

3 - Colorectal cancer risk factors

The risk of developing CRC depends on a number of factors involving an interaction between genetic and environmental causes. Age is the most common risk factor, increasing from the fourth to the eighth decade of life (Haggar and Boushey, 2009). A personal history of CRC or adenoma and chronic inflammatory bowel disease (such as ulcerative colitis and Crohn's disease) are also significant risk factors (Eaden et al, 2001; Dyson and Rutter, 2012; Levi et al, 2012; Martinez et al, 2012; Beaugerie et al, 2013).

Individuals with a family history of CRC also present an increased risk of developing the disease, especially if the index case is diagnosed before 45 years of age (Hemminki and Chen, 2004; Taylor et al, 2010; Cirillo et al, 2013). It is estimated that about 10% of CRC cases are hereditary, occurring predominantly in the context of Lynch syndrome (also designated Hereditary Non-Polyposis Colorectal Cancer, HNPCC), Familial Adenomatous Polyposis (FAP), MUTYH-associated polyposis (MAP), and the hamartomatous polyposis syndromes (Peutz-Jeghers syndrome, juvenile polyposis, and Cowden disease) (Lynch et al, 1993; Al-Tassan et al, 2002; Gammon et al, 2009; Half et al, 2009; Bogaert and Prenen, 2014).

Several studies have shown that migrant populations tend to acquire the relative risk of developing CRC of their adopted countries. Furthermore, inter-regional differences in incidence of CRC, including differences among population groups living in geographic proximity but with different lifestyles, strongly suggest that environmental factors play an important role in the etiology of this disease (Center et al, 2009; Durko and Malecka-Panas, 2014). Several studies have demonstrated that consumption of high amounts of fat, red and processed meat and low intake of fiber, fruits, vegetables, and whole grains might be associated with the development of CRC (van Duijnhoven et al, 2009; Chan e al, 2011). Additionally, has been described

an association between obesity, tobacco and excessive alcohol consumption and the risk of developing CRC (Dai et al, 2007; Paskett et al, 2007; Bongaerts et al, 2008). On the other hand, physical, occupational or recreational, activity appears to lessen the risk of this cancer, probably due to its effect on intestinal transit, bile acid metabolism and immune system (Chao et al, 2004; Wei et al, 2004).

4 - Colorectal cancer diagnosis and staging

CRC screening depends on the risk of the individual. In the general population routine CRC screening should begin at age 50; however, individuals presenting an increased risk due to a personal or family history of CRC or adenomas, inflammatory bowel disease, or a hereditary CRC syndrome require a more personalized screening. CRC screening can be performed by endoscopic, radiologic imaging and stool/fecal-based exams, being colonoscopy the most complete procedure allowing examination of the entire large bowel and simultaneous removal of polyps for histologic analysis (Levin et al, 2008; Czito and Willett, 2012; NCCN, 2013).

Although CRC patients often present minimal or no symptoms, these can include rectal bleeding, bowel dysfunction as constipation or diarrhea, abdominal discomfort, weight loss, fatigue and anemia. The symptoms can differ according to large bowel tumor location. Cancers of the proximal colon are commonly associated with iron-deficiency anemia due to occult blood loss, whereas distal colon cancers are often accompanied by obstruction and rectal bleeding (Hamilton and Sharp, 2004; Astin et al, 2011; Czito and Willett, 2012).

Accurate CRC histology and staging, including differentiation, lymphatic invasion, and extension of tumor free surgical resection margins, is imperative for the determination of diagnosis, therapy and prognosis. The majority (>90%) of large bowel tumors are adenocarcinomas, but can include other histologic types as squamous cell carcinomas, carcinoids, leiomyosarcomas, gastrointestinal stromal tumors and lymphomas (Bosman et al. 2010). The standard CRC staging system is the tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) (Table 1). The grading system for colorectal adenocarcinomas is based on

architectural and cytologic features, but the degree of gland formation is widely considered the most important feature and generally it is classified in grade 1 (well differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated).

Subsite CRC localization analysis in some populations indicates that approximately 60% of them occur in the distal colon, with 30% to 35% occurring in the rectum, 20% to 25% in sigmoid, 3% to 5% in descending colon, 7% to 15% in transverse colon and 25% to 26% in ascending colon (including cecum) (Bresalier 2010; Larsen and Bray, 2010; Cancer Research UK, 2014)

Table 1 - American Joint Committee on Cancer. Tumor, Node, Metastasis (TNM) staging system for colorectal cancer (2009).

Primary Tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through the muscularis propria into pericolic tissues
T4a	Tumor penetrates to the surface of the visceral peritoneum
T4b	Tumor is adherent to or directly invades other organs or structures
Regional Lymph Nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in one to three regional lymph nodes
N1a	Metastasis in one regional lymph node
N1b	Metastasis in two to three regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis
N2	Metastasis in four or more regional lymph nodes
N2a	Metastasis in four to six regional lymph nodes
N2b	Metastasis in seven or more regional lymph nodes
Distant Metastasis (M)	
MX	Distant metastasis cannot be assessed.
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ or site (e.g., liver, lung, ovary, nonregional node)
M1b	Metastases in more than one organ/site or the peritoneum

5 - Colorectal cancer treatment

The mainstay therapy for locoregional colon and rectal carcinoma is surgery. In colon cancer, adjuvant chemotherapy is administered to reduce the risk of recurrence and, according to National Comprehensive Cancer Network (NCCN) guidelines, 5-Fluorouracil (5-FU) regimens in combination with leucovorin or oxaliplatin (FOLFOX) is the current choice for stage III and high-risk stage II patients. In rectal cancer, neoadjuvant combined-modality therapy, including chemotherapy and radiation, is administered to improve resectability, sphincter preservation, and reduce local as well as distant recurrence. Postoperative oxaliplatin-containing regimens such as FOLFOX are typically used in the setting of rectal cancer postoperatively. In the setting of metastatic CRC, combination of 5-FU chemotherapy regimens with an inhibitor of vascular endothelial growth factor (VEGF), such as Bevacizumab, or an inhibitor of epidermal growth factor receptor (EGFR), such as Cetuximab or Panitumumab, is the treatment of choice. EGFR inhibitors should only be used in patients that present *KRAS*/*NRAS* wild-type tumors.

6 - Colorectal carcinogenesis

In 1990, Fearon and Vogelstein described a multistep genetic model that consisted in the accumulation of genetic mutations in multiple genes that regulate cell growth and differentiation, by which colon cancers were believed to progress through an adenoma-carcinoma sequence (Fearon and Vogelstein, 1990). According to this model, CRC arises as a result of mutational activation of oncogenes combined with mutational inactivation of tumor suppressor genes, and the accumulation of genetic alterations, rather than their order, is the responsible for the biological behavior of the cancer. This linear model has evolved to a more complex, comprehensive, and mechanistic approach designated as the chromosomal instability (CIN) pathway. Since then, at least two other major distinct molecular pathways have been recognized, the microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP) pathways (Ionov et al, 1993; Toyota et al, 1999).

In the progression from adenoma to carcinoma, tumors following the MSI and CIN pathways often display mutations in either *APC* or *CTNNB1* (β-catenin) and in

KRAS in the early stages of colorectal carcinogenesis, diverging their genetic and epigenetic profiles more significantly towards malignancy (Ilyas et al, 1999; Miyaki et al, 1999; Jass et al, 2003; Huang et al, 2004). Furthermore, although the subsequent gene targets may be different, mutations frequently affect the same pathways. Several signaling pathways implicated in cell cycle control, such as TGFβ, MAPK, WNT and AKT, are affected in most CRC. For instance, the TGFβ signaling pathway, involved in cell proliferation inhibition, can be compromised in the MSI and CIN pathways through *TGFBR2* and *SMAD* gene alterations, respectively (Miyaki et al, 1999; Roth et al, 2000; Shin et al, 2000).

6.1 - Chromosomal instability pathway

CIN is the most common cause of genomic instability in CRC, accounting for 70-85% of sporadic CRC. This pathway is characterized by the occurrence of aneuploidy, chromosomal genomic amplifications, and a high frequency of loss of heterozigosity (LOH). Chromosomal structural alterations occurs more frequently in chromosomes 1, 5, 8, 17, and 18, presenting frequently loss of the long arm of chromosome 5 (5q) and 18 (18q) and the short arm of chromosome 17 (17p). Mutations in oncogenes and tumor suppressor genes (e.g. *APC*, *KRAS*, *TP53*) essential for colorectal carcinogenesis are also observed at a high frequency (Smith et al, 2002; Lüchtenborg et al, 2005; Camps et al, 2006; Diep et al, 2006).

APC is a tumor suppressor gene located in 5q21 that is mutated in the germline in the CRC hereditary syndrome FAP (Nishisho et al, 1991). The most well documented function of APC is in the canonical Wingless-Type MMTV Integration Site Family (WNT) signaling pathway (Morin et al, 1997). APC induces degradation of β-catenin, functioning as a negative regulator of the WNT, pathway controlling cell growth, differentiation and apoptosis. APC constitutes with AXIN and GSK-3β a multiprotein complex that bounds to β-catenin and causes its phosphorylation, subsequent ubiquitination, and destruction in the proteosome. The disruption of this complex leads to the accumulation in the cytoplasm of a stabilized, free β-catenin that can translocate to the nucleus and lead to the transcription of genes involved in cell cycle regulation (Saito-Diaz et al, 2013). In CRC, one of the main causes of disruption

of this multiprotein complex is mutation of the *APC* gene, which is an early event in colorectal carcinogenesis with frequencies of 30 to 70% in sporadic colorectal adenomas and 34% to 80% in sporadic CRC (Miyoshi et al, 1992; Powell et al, 1992; Yashima et al, 1994; Rowan et al, 2000; Diergaarde et al, 2003; Lüchtenborg et al, 2004). In sporadic CRC cases with wild-type *APC* gene, *APC* gene promoter hypermethylation or *CTNNB1* point mutation have been described to explain the sustained activation of the WNT signaling pathway (Sparks et al, 1998; Thorstensen et al, 2005; Sameer et al, 2011).

KRAS is a proto-oncogene located at 12p12.1 that encodes a GTP-binding protein. When it is bound to GTP, the ras protein is active and becomes inactive by hydrolysis of GTP to GDP. Mutations in this gene result in a complex less sensitive to hydrolysis, keeping the ras protein in a constitutively active state. This will lead to cell proliferation through a variety of signaling pathways, including the mitogen-activated protein kinases (MAPK) pathway (Maruta et al, 1994; Guerrero et al, 2000). The frequency of mutations in the *KRAS* proto-oncogene in sporadic CRC is 30 to 50% (Smith et al, 2002; Calistri et al, 2005; Berg et al, 2010).

18q deletion, encompassing the *SMAD2* and *SMAD4* genes, has been reported in up to 70% of CRC (Miyaki et al, 1999; Woodford-Richens, 2001). SMAD proteins mediate the TGFβ signaling pathway, which regulates cell growth and apoptosis (Zawel et al, 1998; Zhou et al, 1998; Kouvidou, 2006). About 20% of CRCs have been found to present *SMAD4* mutations, which occur at a later stage of colorectal carcinogenesis and are an indicator of advanced phenotypes (Miyaki et al, 1999; Woodford-Richens, 2001).

TP53 is a tumor suppressor gene located on the short arm of chromosome 17 (17p13.1) that regulates cell cycle and is involved in apoptosis and DNA repair (Isobe, 1986). The protein encoded by this gene is a key transcription factor that mediates cell cycle arrest in order to facilitate DNA repair during replication or induces apoptosis if the repair is not possible (Green and Kroemer, 2009). *TP53* loss, usually by 17p deletion, is described as a late event in the CIN pathway, being associated with the adenoma to carcinoma transition. This fact is supported by the observed increase in alterations frequency, either by mutation or by LOH, with the histological

stage of the lesion, namely 4 to 14% in adenoma and 27 to 61% in CRC (De Benedetti et al, 1993; Ohue et al, 1994; Hardingham et al, 1998; Smith et al, 2002; Aissi et al, 2014; Vasovcak et al, 2014).

Although recent studies indicate that simultaneous alterations in these genes is not as frequent as initially believed and different molecular patterns have been observed in CRC, that develop through the CIN pathway, mutations in *APC*, *KRAS* and *TP53* genes are essential events in colorectal carcinogenesis and this model of carcinogenesis remains a paradigm (Smith et al, 2002; Calistri et al, 2005).

6.2 - Microsatellite instability pathway

Another important pathway that leads to genomic instability is the MSI pathway that is operative in 15% to 20% of sporadic CRC and in as up to 96% of Lynch syndrome CRC (Lothe et al, 1993; Thibodeau et al, 1993; Aaltonen et al, 1994; Bocker et al, 1996, Caldés et al, 2004; Mead et al, 2007). Microsatellites are short, repetitive nucleotide sequences consisting of 1 to 6 bases per repeat unit that are distributed throughout the human genome. These sequences are more prone to errors during DNA replication due to their repetitive structure (Farber et al, 1994; Arzimanoglou et al, 1998). The DNA mismatch repair (MMR) system function is to recognize and repair base-pair mismatches that occur during DNA replication and that have escaped the proofreading process, increasing the replication fidelity 100 to 1000-fold. Microsatellite instability is a reflection of the inability of the MMR system to correct these errors (Koi et al, 1994; Umar et al, 1997).

The MMR system is a multi-protein complex composed by MSH2, MSH6 and MSH3 (human MutS homologs (MSH) of Escherichia coli), MLH1 and MLH3 (human MutL homologs (MLH) of Escherichia coli) and PMS2 (post-meiotic segregation MutL homolog of Escherichia coli). These proteins contain heterodimeric interaction domains, ATP binding/hydrolysis domains and protein–protein interaction domains. When DNA structural integrity is compromised, the first step of eukaryotic mismatch repair is error recognition by MSH2 and heterodimer formation with either MSH3 or MSH6, depending on the nature of the irregularity. MSH3 is specific for insertion/deletion loops of 2 to 4 nucleotides, while MSH6 is specific for single

nucleotide loops or mismatches. The next step is the recruitment of the MLH family proteins, namely MLH1 and PMS2, by the ATP-bound MSH heterodimer. The multiprotein complex will displace the main processive DNA polymerase and the sliding clamp PCNA, and recruit base excision machinery to remove the tract in which the mismatch occurred. The final step is resynthesis of the excision gap by the replicative DNA polymerase using the remaining DNA strand as a template (Figure 3) (Peltomaki, 2001a, 2001b; Marti et al, 2002). Germline mutations in one of the MMR genes, namely in tumors from patients with Lynch syndrome, or epigenetic alterations such as *MLH1* promoter hypermethylation in sporadic CRC, are the most common cause of MSI (Cunningham et al, 1998; Forster et al, 1998; Herman et al, 1998; Kuismanen et al, 2000; Wheeler et al, 2000).

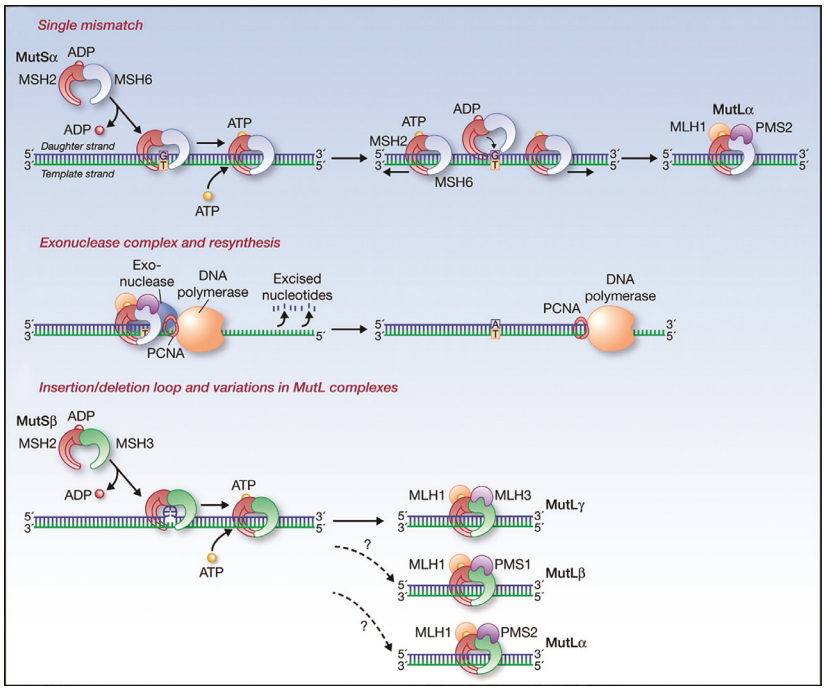

Figure 3 - Steps involved in mismatch repair during DNA replication. Different types of mismatches, single base mismatches or insertion/deletion of 2 to 4 bases, are recognized by MSH2-MSH6 heterodimer or MSH2-MSH3 heterodimer, respectively. A sliding clamp is created around the DNA, requiring the exchange of ATP for ADP. This complex is then bound by heterodimers of MLH1 with PMS2 or PMS1 or MLH3. Excision of the mismatch occurs when the DNA MMR protein sliding clamp interacts with exonuclease-1, PCNA, and DNA polymerase. The strand containing the mismatch is excised and resynthesis occurs with correction of the error (adapted from Sinicrope and Sargeant, 2012).

Sporadic MSI CRC tends to be diploid and present lower frequency of LOH and mutations in *APC*, *KRAS* and *TP53* genes (Olschwang et al, 1997; Forster et al, 1998; Salahshor et al, 1999; Jass et al, 2003; Jones et al, 2005). Contrarily, mutations in the *BRAF* gene have been identified in sporadic CRC with MSI associated with hypermethylation of the *MLH1* promoter gene (Wang et al, 2003; Domingo et al, 2004). All point mutations occur within the kinase domain of *BRAF*, the most frequent being the p.Val600Glu mutation, leading to the constitutive activation of Braf kinase (Davies, 2002; Wang et al, 2003). *BRAF* is a proto-oncogene member of the RAF family of serine/threonine kinases and is an immediate downstream effector of RAS in the RAS/RAF/MAPK pathway. *BRAF* mutation is almost mutually exclusive with *KRAS* mutations (Domingo et al, 2004; Koinuma et al, 2004).

Furthermore, through the MSI pathway, colorectal cancer progression is accelerated by a rapid mutation accumulation in coding repetitive sequences of target genes with growth-related functions (Malkhosyan et al, 1996; Perucho, 1996). These frameshift mutations will lead to the inactivation of the function of these genes, giving them a potential role in colorectal carcinogenesis (Iacopetta et al, 1998; Woerner et al, 2001; Samowitz et al, 2002). There is a large list of genes containing coding repeats that are susceptible to mutations in the presence of a defective MMR system. For example, genes involved in DNA repair (e.g. *MSH3*, *MSH6* and *MLH3*), apoptosis (e.g. *BAX*, *BCL10*, and *CASP5*), signal transduction (e.g. *TGFBR2*, *ACVR2A*, *IGF2R*) and cell cycle regulation (*PTEN* and *PRDM2*). Inactivation of the TGFβ signaling pathway appears to play a key role in MSI CRC development, resulting in uncontrolled cellular proliferation and dysregulation of cell death mechanisms (Derynck et al, 2001; Elliott et al, 2005). The TGFβ superfamily of proteins includes three TGF-β isoforms, the bone morphogenetic proteins (BMP), inhibins and activins. In MSI CRC, the most frequent mutated members of this pathway are *TGFBR2* (70 to 90%) and *ACVR2A* (83% to 85%), occurring frequently in concomitance (Iacopetta et al, 1998; Schwartz et al, 1999; Calin et al, 2000; Jung et al, 2004; Fernández-Peralta et al, 2005; Jung et al, 2006; Ogino et al, 2007; Shima et al, 2011). Genes with a role in apoptosis are also frequently mutated in MSI CRC. The pro-apoptotic tumor suppressor gene *BAX* is the canonical example, presenting mutations in 30 to 66 %

of MSI CRC (Schwartz et al, 1999; Calin et al, 2000; Samowitz et al, 2002; Potočnik et al, 2003; Fernández-Peralta et al, 2005; Jung et al, 2006; Shima et al, 2011). These frameshift mutations will lead to loss of function of *BAX*, promoting cell escape from intrinsic apoptosis mechanisms. Somatic mutations in microsatellite sequences within *MSH3* and *MSH6* have also been described in sporadic MSI CRC with frequencies ranging from 20 to 46% and 17.5% to 40%, respectively (Malkhosyan et al, 1996; Schwartz et al, 1999; Duval et al, 1999; Orimo et al, 1999; Calin et al, 2000). These mutations have been considered as secondary events and *MSH3* and *MSH6* as “secondary mutators” in a "mutator that mutates another mutator" model, given that they are DNA repair proteins prone to frameshift mutations themselves; when mutated somatically the MSI phenotype is exacerbated (Orimo et al, 1999).

Sporadic MSI CRC present a number of characteristic clinicopathologic features. CRC tend to be located more proximally in the colon, be poorly differentiated and of the mucinous histology type, display extensive lymphocytic infiltration and present a better overall survival compared with microsatellite stable tumors (Senba et al, 1998; Samowitz et al, 2001; Smyrk et al, 2001; Gervaz et al, 2002).

6.3 - CpG island methylation pathway

The observation of a widespread CpG island methylation in CRC lead to the discovery of a third pathway, designated the CpG Island Methylation Pathway (CIMP) (Toyota et al, 1999). Methylation of gene promoter region results in gene silencing, providing an alternative mechanism for loss of function of tumor suppressor genes. The CIMP pathway is present in approximately 20% of patients with CRC. Genes implicated in colorectal carcinogenesis that can be silenced by hypermethylation include *APC*, *MGMT* and the classical example of the hypermethylation silencing of *MLH1*, which occurs in >80% of sporadic MSI CRC (Wheeler et al, 2000; Arnold *et al*, 2004). Five markers have been indicated to serve as markers for CIMP, *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* and CIMP positivity is defined by methylation of at least three markers (Weisenberger et al, 2006). It has been suggested that CIMP positive tumors can be divided in two types, CIMP high related to *BRAF*

mutations and *MLH1* promoter hypermethylation and CIMP low related to *KRAS* mutations (Kambara et al, 2004; Shen et al, 2007; Curtin et al, 2011).

CIMP CRC usually develops through an alternative route of colorectal cancer carcinogenesis denominated the serrated pathway in which the precursor lesions are sessile serrated adenomas (Kambara et al, 2004). CIMP CRC tumors present particular clinicopathological features, although some of them overlap with the ones observed in MSI CRC, such as an association with proximal colon location and poor differentiation. Additionally, these patients usually are female and of more advanced age, with a history of cigarette smoking (Grady, 2007; Ogino, 2009).

Several studies indicate that the three pathways, (CIN, MIN and CIMP) are not mutually exclusive, being possible that tumors can exhibit features of more than one or even follow yet uncharacterized pathways. CRC subtyping has been addressed by several authors regarding prognosis, biological, morphological and clinical features showing a clear evidence for the presence of CRC molecular subtypes (Kim et al, 2013; Samadder et al, 2013). Accordingly to Jass (2007), using pathological, clinical and molecular features, CRC should be classified in to five molecular subtypes: CIMP high/MSI high (12% of CRC), with origin in serrated adenomas and characterized by *BRAF* mutation and *MLH1* promoter hypermethylation; CIMP high/MSI low or microsatellite stable (8%), with origin in serrated adenomas and characterized by *BRAF* mutation and methylation of multiple genes; CIMP low/MSI low or microsatellite stable (20%), with origin in tubular, tubulovillus, or serrated adenomas and characterized by chromosomal instability (CIN), *KRAS* mutation, and *MGMT* methylation; CIMP negative/microsatellite stable (57%), which originates in traditional adenoma and is characterized by CIN; and Lynch syndrome, CIMP negative/MSI high and negative for *BRAF* mutations. According to this classification approximately 70% of CRC arise via a adenoma pathway and approximately 30% via a serrated pathway, characterized by activation of the MAPK pathway (*KRAS* or *BRAF* mutations, mutually exclusive), presence of CIMP (Low or High) and presenting or not MSI.

7 - Lynch syndrome

7.1 - Lynch syndrome characterization

The most common cause of hereditary predisposition to CRC is Lynch syndrome, accounting for up to 4% of all CRC (Aaltonen et al, 1998; Barnetson et al, 2006; Hampel et al, 2008). Lynch syndrome is an autosomal dominant disorder characterized by a high incidence of early-onset (<50 years old) CRC and extracolonic tumors of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary, and hepatobiliary tract (Lynch et al, 2003). The diagnostic criteria for identifying individuals with Lynch syndrome were established in 1991 in Amsterdam. These criteria, named Amsterdam I Criteria, stated the following: (1) three relatives with colorectal cancer, one being a first-degree relative of the other two; (2) two successive generations affected with colorectal cancer; (3) one family member who developed colorectal cancer before age 50 and (4) FAP must be excluded and all cancers verified pathologically (Vasen et al, 1999). However, these criteria underestimate Lynch syndrome in some families, so the Amsterdam II Criteria were developed in order to include extracolonic tumors with the goal of increasing sensitivity. However, although family history alone may be used to highlight high-risk families, several studies demonstrated that these criteria still remained too restrictive. A broader set of criteria known as the revised Bethesda guidelines were developed, combining histopathologic and/or genetic analysis with family history (Table 2) (Umar et al, 2004). Patients that meet only the Bethesda guidelines should first have their tumors assessed for MSI and/or MMR protein staining by immunohistochemistry (IHC). In order to discriminate between a hereditary or a sporadic event, tumors with immunohistochemical loss of MLH1 should be analyzed for *MLH1* promoter hypermethylation and *BRAF* (p.Val600Glu hotspot mutation) (Wang et al, 2003; Domingo et al, 2004, 2005; Miyaki et al, 2004).

Table 2 - Amsterdam criteria II and Revised Bethesda guidelines (adapted from Vasen et al, 2007).

Amsterdam criteria II
1. At least three relatives with CRC or with a Lynch syndrome-associated cancer[1],
2. one relative should be a first-degree relative of the other two,
3. two successive generations should be affected,
4. one tumor should be diagnosed before the age of 50 years,
5. FAP should be excluded in the CRC case if any,
6. tumors should be verified by histopathological examination.
Revised Bethesda guidelines
1. CRC diagnosed in a patient aged <50 years.
2. Presence of synchronous, metachronous colorectal or other Lynch syndrome-related tumors, regardless of age.
3. CRC with MSI-H phenotype diagnosed in a patient aged <60 years.
4. Patient with CRC and a first-degree relative with a Lynch syndrome-related tumor[2], with one of the cancers diagnosed at age <50 years.
5. Patient with CRC with two or more first-degree or second-degree relatives with a Lynch syndrome-related tumor regardless of age.

[1]Lynch syndrome-associated cancer includesthose of endometrium, small bowel, ureter or renal pelvis.

[2]Lynch syndrome-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter, renal pelvis, biliary tract and brain tumors, sebaceous gland adenomas and keratoacanthomas, and carcinoma of the small bowel.

Lynch syndrome phenotypic features include early-onset colorectal cancer, synchronous or metachronous CRC, and a predominance of right-sided tumors. Additionally, the patients present a comparatively favorable prognosis and absence of distant organ metastasis, with CRC presenting dense lymphocytic infiltrates pointing to a possible protective effect by the immune system (Percesepe et al, 1997; Peel et al, 2000; Smyrk et al, 2001).

7.2 - MMR germline mutational spectrum

Lynch syndrome is caused mainly by mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* and, as indicated above, MMR mutation carriers present a substantially increased risk of colorectal and endometrial cancers, along with increased risk of ovarian, gastric, small bowel, urothelial, brain, hepatobiliary, pancreatic, bladder, kidney, prostate and breast cancer (Lynch et al, 2003, Peltomäki et al, 2005).

Identification of the underlying germline mutation guides clinical management of Lynch syndrome families, with implications for presymptomatic surveillance and cancer treatment options. Molecular genetic heterogeneity must be considered when assessing the Lynch syndrome cancer phenotype. Current data suggest that families with *MLH1* mutations have a higher expression of CRC, families with *MSH2* mutations present more extra-colonic cancers and families that harbor *MSH6* mutations develop CRC at a more advanced age and have a higher risk of developing endometrial cancers (Lin et al, 1998; Wagner et al, 2001; Koornstra et al, 2009).

As of December 2012, according to the International Society for Gastrointestinal Hereditary Tumors (InSiGHT) database, of the 2,360 constitutional variants described 39% were in the *MLH1*, 36% in *MSH2*, 19% in *MSH6* and 6% in *PMS2* genes (Thompson et al, 2014). Recently, germline deletions in the *EPCAM* gene (a gene located directly upstream of *MSH2*), which encodes a epithelial cell adhesion molecule, has been identified as the causative mutation in some families with Lynch syndrome operating by epigenetic silencing *MSH2* (Ligtenberg et al, 2009).

The mutational spectrum of MMR genes includes nonsense/frameshift variants predicted to cause protein truncation (34%), nonsynonymous variants (32%) including missense substitutions, small in-frame insertion-deletion mutations and read-through alterations of the translation termination codon, intronic variants (11%), splicing site alterations (7%), synonymous variants (5%), and ATG/UTR variants (2%) (Thompson et al, 2014). Large genomic rearrangements represent 9% of the reported variants, consisting of single or multi-exonic deletions or duplications, but its frequency among Lynch syndrome families can be as high as 20% in certain populations, mainly occurring in the *MSH2* and *MLH1* genes (Charbonnier et al, 2002; Di Fiore et al, 2004; Thompson et al, 2014). Three major mechanisms have been proposed for genomic rearrangements in the human genome: nonallelic homologous recombination (NAHR), non-homologous end-joining (NHEJ) and the fork stalling and template switching (FoSTeS) models (Gu et al, 2008; Chen et al, 2010). Molecular characterization of large rearrangement breakpoints demonstrated that the majority are caused by homologous recombination between Alu repeats. Alu repeats are short

interspersed elements whose transposition has been repeatedly implicated in genetic variability and heritable disorders, including Lynch syndrome and hereditary breast/ovarian cancer (Moisio et al, 1996; Peixoto et al, 2009).

Although Lynch syndrome is characterized by marked genetic heterogeneity, considering the several types of mutations located throughout the four relevant MMR genes, the occurrence of recurrent and/or founder effects explain why some specific mutations are observed at high frequency in well-defined populations or ethnic groups. Founder mutations have been identified in Lynch families worldwide (Chan et al, 2001; Wagner et al, 2003; Caluseriu et al, 2004; Sun et al, 2005). For example, the Finnish founder mutation *MLH1* exon 16 deletion with an age estimation of 400 to 1075 years, accounts for more than 50% of all Lynch syndrome families in certain regions of Finland (Moisio et al, 1996). On the other hand, the *MSH2* splice-site mutation c.942+3A>T, which was proven to be widespread in the Newfoundland population through a founder effect, has been observed in many other populations arising *de novo*, and therefore is considered a worldwide recurrent mutation with a founder effect in Newfoundland (Froggatt et al, 1999; Desai et al, 2000). This mutation apparently arises repeatedly *de novo* because the region where it occurs, a microsatellite sequence, is susceptible to slippage during DNA replication, accounting for 5 to 10% of all Lynch syndrome families worldwide (Desai et al, 2000; Hampel et al, 2005). The identification of founder and/or recurrent mutations facilitates the molecular diagnosis of Lynch syndrome by making cost-effective mutational analysis of specific gene regions before full screening of all MMR genes.

7.3 - Lynch syndrome CRC somatic alterations

More than 95% of CRC from Lynch syndrome patients present MSI, making it a dependable indicator of MMR deficiency and providing a straightforward, although indirect, approach to identify MMR mutation carriers (Aaltonen et al, 1994; Dietmaier et al, 1997). In 1997, the National Cancer Institute recommended a panel of 5 microsatellite loci, including two mononucleotide (BAT25, BAT26) and three dinucleotide markers (D2S123, D5S346, and D17S250), to assess the MSI tumor status (Boland et al, 1998). Subsequently, other researchers proposed a panel with

five mononucleotide repeat markers (BAT25, BAT26, NR21, NR24, and NR27) that obviates the need for normal tissue for comparison and presents a higher sensitivity and specificity (Patil et al, 2012). Depending on the number of microsatellite markers displaying novel alleles, MSI can subsequently be classified as MSI-H (MSI-H, >2 out of 5 markers), MSI-L (MSI-L, 1 out of 5), or microsatellite stable (MSS, 0 out of 5) (Boland et al, 1998). The presence of MSI-H is normally associated with mutations in *MLH1* and *MSH2* genes and MSI-L appears to some extent due to mutations in the *MSH6* gene (Peterlongo et al, 2003; Barnetson et al, 2006). Whether MSI-L CRC constitutes a separated group of tumors is still not clear: whereas some authors suggest that they do not present clear differences from MSS CRC, regarding clinical, biological, and morphological parameters, others defend that they constitute a separate group of tumors based in the observation of distinct molecular profiles when compared to MSI-H and MSS CRC (Whitehall et al, 2002; Yearsley et al, 2006).

Although sporadic and Lynch syndrome MSI CRC share certain clinicopathologic and molecular characteristics, some studies indicate differences between these two groups. Lynch syndrome MSI CRC generally occur at a younger age and present more frequently lymphocytic infiltration and a mucinous histologic type (Young et al, 2001; Shia et al, 2003; Yearsley et al, 2006). Furthermore, even though the tumors tend to occur more frequently in the proximal colon, some authors have reported frequencies of to 40% of Lynch syndrome MSI CRC occurring in distal colon, contrasting with sporadic MSI CRC of which ~90% occur in the proximal colon (Kim et al, 1994; Mueller-Koch et al, 2005; Moghbeli et al 2011; Moussa et al, 2011). Significant differences in the spectrum of molecular alterations between MSI CRC occurring in Lynch syndrome and in sporadic CRC have also been observed. *CTNNB1* gene mutations occur in MSI Lynch syndrome CRC but not in sporadic MSI CRC (Johnson et al, 2005). Inversely, *BRAF* mutations, namely p.Val600Glu, are associated with sporadic MSI CRC (Wang *et al*, 2003; Domingo *et al*, 2004, 2005). The differences in the molecular profiles of these two pathways are consistent with the differing carcinogenesis routes of hereditary *versus* sporadic MSI CRC, specifically the traditional adenoma-carcinoma sequence in the former and a serrated route in the latter.

In MSI CRC from patients with Lynch syndrome, similarly to sporadic MSI CRC, mutations have been described in coding microsatellite sequences of several genes with important cellular roles, such as growth factor receptors (*TGFBR2* and *IGF2R)*, genes involved in apoptosis (*BAX*), as well as genes relevant for DNA repair (*MSH3* and *MSH6*) (Perucho, 1996; Boland et al, 1998; Yamamoto et al, 1998; Yagi et al, 1998; Fujiwara et al, 1998; Calin et al, 2000). However, differences between the MSI profiles in different types of Lynch syndrome associated cancers have been described, for instance between endometrial and colorectal cancers. This suggests that biological features and functional roles of target genes may differ depending on the tissue of tumor origin (Myeroff et al, 1995; Duval et al, 1999, Kuismanen et al, 2002). Furthermore, differences have been reported between distal and proximal sporadic MSI CRC regarding target genes mutational spectrum (Pinheiro et al, 2010). In Lynch syndrome, there is virtually no data relating target gene mutation frequency or pattern with CRC large bowel location.

AIMS

This study aimed to ascertain the relative contribution of the underlying molecular defect driving carcinogenesis, on one hand, and the site of tumor origin, on the other, for the pattern of acquired genetic changes in CRC from patients with Lynch syndrome. Specifically, the objectives of this thesis were:

1. To perform haplotype analyses in Portuguese Lynch syndrome families presenting a frequent novel exonic rearrangement affecting *MLH1* gene, in order to determine if this is a founder mutation.
2. To perform haplotype analyses in Portuguese and worldwide Lynch syndrome families presenting the frequent *MSH2* c.388_389del mutation, in order to determine if it is a founder and/or a recurrent mutation.
3. To characterize and compare the spectrum of somatic mutations in selected target genes involved in colorectal carcinogenesis in CRC from patients with Lynch syndrome with different germline MMR mutations.
4. To characterize and compare the pattern of somatic mutations and disrupted pathways in CRC arising in proximal and distal colon in Lynch syndrome patients.

PAPER I

Manuela Pinheiro, Carla Pinto, Ana Peixoto, Isabel Veiga, Bárbara Mesquita, Rui Henrique, Manuela Baptista, Maria Fragoso, Olga Sousa, Helena Pereira, Carla Marinho, Luis Moreira Dias, Manuel R. Teixeira.

"A novel exonic rearrangement affecting *MLH1* and the contiguous *LRRFIP2* is a founder mutation in Portuguese Lynch syndrome families"

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ARTICLE

A novel exonic rearrangement affecting *MLH1* and the contiguous *LRRFIP2* is a founder mutation in Portuguese Lynch syndrome families

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Purpose: Although Lynch syndrome is characterized by marked genetic heterogeneity, some specific mutations are observed at high frequency in well-defined populations or ethnic groups due to founder effects. **Methods:** Genomic breakpoint identification, haplotype analysis, and mutation age determination were performed in 14 unrelated patients and 95 family members presenting the same *MLH1* exonic rearrangement, among a series of 84 Lynch syndrome families with germline mutations in *MLH1*, *MSH2,* or *MSH6*. **Results:** All 14 probands harbored an identical deletion, comprising exons 17–19 of the *MLH1* gene and exons 26–29 of the *LRRFIP2* gene, corresponding to the *MLH1* mutation c.1896 + 280_oLRRFIP2: c.1750-678del. This mutation represents 17% of all deleterious mismatch repair mutations in our series. Haplotype analysis showed a conserved region of approximately 1 Mb, and the mutation age was estimated to be 283 ± 78 years. All 14 families are originated from the Porto district countryside. **Conclusion:** We have identified a novel *MLH1* exonic rearrangement that is a common founder mutation in Lynch syndrome families, indicating that screening for this rearrangement as a first step may be cost-effective during genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the Porto district. ***Genet Med* 2011:13(10):895–902.**

Key Words: *MLH1, LRRFIP2, exonic rearrangement, founder mutation, Lynch syndrome*

Lynch syndrome is a highly penetrant, autosomal dominant disease characterized by early-onset colorectal cancer (CRC) and extracolonic tumors of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary, and hepatobiliary tract.[1] Families are usually selected for genetic testing using the Amsterdam criteria or the Bethesda guidelines.[2,3] Although the former are rather specific and allow selection of families for direct germline mutation analysis, the latter have higher sensitivity but lower specificity and require a prescreening by microsatellite instability analysis or immunohistochemistry for mismatch repair (MMR) proteins in tumor tissue.[4,5]

The genetic defect underlying Lynch syndrome is a germline mutation in one of the four MMR genes *MLH1*, *MSH2*, *MSH6,* and *PMS2*.[6] Approximately 85% of the mutations described are found in *MSH2* and *MLH1*, with *MSH6* and *PMS2* mutations accounting for the remaining 10% and 5%, respectively (International Collaborative Group on HNPCC Mutation Database, http://www.insight-group.org). The mutational spectrum of Lynch syndrome includes mainly point mutations, small insertions, and deletions, as well as changes affecting splice sites. However, the use of new techniques allowed the discovery that a significant proportion of pathogenic alterations are large genomic rearrangements, in most cases single or multiexonic deletions or duplications inactivating *MLH1* or *MSH2*.[7] Based on the October 2009 Human Gene Mutation database, *MLH1* and *MSH2* exonic deletions/duplications represented 21% of all reported mutations.[8]

Although Lynch syndrome can be originated by many different mutations located throughout the four relevant MMR genes, specific mutations are observed at high frequency in well-defined populations or ethnic groups due to founder effects. For example, founder mutations have been identified in Lynch syndrome families from China, the United States, Italy, and among Ashkenazi Jews.[9–12] The identification of founder mutations facilitates the molecular diagnosis of Lynch syndrome by making cost-effective mutational analysis to specific gene regions before full screening of all MMR genes. We herein present a novel *MLH1* exonic rearrangement that is a founder mutation in Portuguese Lynch syndrome families.

MATERIALS AND METHODS

Patients, samples, and DNA extraction

This study includes 14 Lynch syndrome families presenting the same *MLH1* exonic rearrangement, from a total series of 84 families with pathogenic *MLH1*, *MSH2,* or *MSH6* germline mutations (data not shown), all of which have been identified by routine genetic diagnosis during the period of 1997 to 2009 at the Genetics Department of the Portuguese Oncology Institute, Porto, Portugal, after genetic counseling and informed consent. Seven families were followed up at the Portuguese Oncology Institute, six at the S. João Hospital, and one at the Padre Américo Hospital, all located in the Porto district. Nine of the 14 families fulfilled the Amsterdam criteria, whereas the re-

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maining presented the Bethesda criteria for genetic testing. After written informed consent, DNA was isolated from peripheral blood samples from the 14 index individuals and subsequently from 95 family members, using the salt–chloroform extraction method.[13] The geographic origin of these families was inferred from the birthplace of the oldest carrier or of the oldest affected family member most likely to be a carrier.

Microsatellite instability and MMR immunohistochemical analyses

In all nine families that fulfilled the Amsterdam criteria and in three of the five cases presenting the Bethesda criteria (because tumor sample was not available), MMR mutation screening was performed directly from the blood sample of the index case. In the remaining two families with Bethesda criteria and available tumor sample, microsatellite instability and MMR immunohistochemical analyses were performed in the carcinoma sample of one index case and on a tubulovillous adenoma from the second index case. Additionally, MLH1 immunoexpression was assessed in four additional carcinomas from three families with Amsterdam criteria (one index case from one family, one index case and one affected relative from a second family, and one affected relative from a third family).

Microsatellite instability evaluation was performed using the Bethesda panel of markers (BAT25, BAT26, D2S123, D5S346, and D17S250) and the 1997 National Cancer Institute guidelines. Polymerase chain reaction (PCR) was carried out as previously described using fluorescence-labeled primers.[14] Fragments were analyzed for length variations on an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, CA), and allele sizes were determined using Genemapper software (version 3.7, Applied Biosystems). The results were independently scored by two observers, and a second round of analyses confirmed the results.

Assessment of MLH1, MSH2, MSH6, and PMS2 immunoexpression was performed as described previously.[15]

Screening for *MLH1* and *MSH2* germline alterations

The 14 index individuals had initially been screened for mutations in *MLH1* and *MSH2* coding exons (except the acceptor splice site of *MLH1* exon 12, *MSH2* exon 1, and the acceptor splice site of *MSH2* exon 5) by denaturing gradient gel electrophoresis (DGGE), using primers and conditions as described by Ingeny (The Netherlands) and Wu et al.[16] Fragments with abnormal DGGE patterns and the acceptor splice site of *MLH1* exon 12, *MSH2* exon 1, and the acceptor splice site of *MSH2* exon 5 were analyzed by direct sequencing in an ABI PRISM 310 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems), according to the manufacturer's recommendations. The 14 index cases and the 95 family members reported in this study were then screened for *MSH2* and *MLH1* exonic deletions and duplications by multiplex ligation-dependent probe amplification (MLPA), according to the instructions of the SALSA MLPA P003 and P248 MLH1/MSH2 kits (MRC-Holland, Amsterdam).

Genomic breakpoint identification

The strategy for breakpoint identification was based on the heterozygosity status information obtained from a set of microsatellite (including the ones used in the haplotype studies, see later) and single-nucleotide polymorphism (SNP) markers (Fig., Supplemental Digital Content 1, http://links.lww.com/GIM/A182). Subsequently, primers were designed spanning the putative breakpoints, and long-range PCR was carried out using the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany), using conditions recommended by the manufacturer. PCR fragments containing the suspected weight were sequenced with BigDye Terminator cycle sequencing chemistry on an ABI PRISM 310 automatic sequencer (Applied Biosystems), according to the manufacturer's recommendations.

The deletion nomenclature is in agreement with the rules recommended by the Human Genome Variation Society (www.hgvs.org/mutnomen). Genomic breakpoint locations are given using the reference sequences NM_000249 and NM_006309 for *MLH1* and *LRRFIP2*, respectively.

Design of mutation-specific assay

After breakpoint identification, we designed a single-nucleotide primer extension assay to detect this *MLH1* rearrangement. First, a three-primer PCR selective amplification was developed in which the mutated allele is amplified with primers MLH1-INT16F 5′-AAATTGATGAGGTGTGACAGCCATTCT-3′ (forward) and LRRFIP2-INT25R 5′-AAGGACAGCTGGGGAAGCCA-3′ (reverse) and the normal allele with the same forward primer and the reverse primer MLH1-INT16R 5′-GGCCTGCAGGGATTCG-GCTC-3′. PCR reactions were performed in a 20 μL reaction containing 30–50 ng of DNA, 2 μL of 10x Taq reaction buffer, 1.5 μL of $MgCl_2$ (1.875 mM), 1 μL of deoxynucleoside triphosphate mix (250 μM deoxythymidine triphosphate, 250 μM dexyadenosine triphosphate, 250 μM deoxyguanosine triphosphate, and 250 μM deoxycytidine triphosphate, Applied Biosystems), 0.2 pmol/μL of primer MLH1-INT16F, 0.1 pmol/μL of primers LRRFIP2-INT25R and MLH1-INT16R, and 0.75 units of Taq DNA polymerase (Fermentas). After a 95°C preincubation step for 10 minutes, PCR was performed in a total of 35 cycles using the following conditions: 95°C denaturation for 30 seconds, annealing at 58°C for 45 seconds, and extension at 72°C during 45 seconds, followed by 10 minutes of final extension at 72°C.

After PCR, we performed a multiplexed nucleotide primer extension reaction using dye label terminators (SNaPshot kit, Applied Biosystems). The primers were designed in the forward direction, with one annealing immediately 5′ to the first nucleotide of the breakpoint region questioning both the wild-type and the mutated alleles (BKP1F 5′-GAGGTAGAAGTTG-CAGTGA-3′) and the second (BKP2-WTF 5′-GACTGACG-TAGAAGTTGCAGTGAGC-3′) and the third primers (BKP2-MTF 5′-GACTGACGTAGAAGTTGCAGTGACC-3′) being specific to the wild-type and mutated alleles, respectively, and questioning the third nucleotide of the breakpoint region (Fig., Supplemental Digital Content 2, http://links.lww.com/GIM/A183). The multiplex primers were designed to be of different lengths using a nonhomologous PolydGACT tail at the 5′ end, so that they could be distinguished by size during capillary electrophoresis separation. The SNaPshot reaction was performed with 3, 2, and 1 pmol/μL of primers BKP1F, BKP2-WTF, and BKP2-MTF, respectively.

Analysis of breakpoints sequence context

Breakpoints were defined as a set of coordinates on the genome spanning the genomic sequence of the deletion. Bioinformatics analyses were carried out to analyze the genomic context of the region. Using the RepeatMasker software, low-complexity DNA sequences and interspersed repeats were searched in both *MLH1* intron 16 and *LRRFIP2* intron 25.

Microsatellite and SNP typing

A total of 14 probands and 95 family members were genotyped for polymorphic microsatellite markers flanking *MLH1*, namely D3S1609, D3S1612, D3S1561, TR89812, D3S1611, TR100328,

D3S1298, D3S3527, and D3S3522. The order of the markers, the consensus repeat, and the distances relative to each other and to *MLH1* are shown in Fig., Supplemental Digital Content 1, http://links.lww.com/GIM/A182. The physical distances of the genetic markers were derived from National Center for Biotechnology Information Map Viewer (http://www.ncbi.nlm.nih.gov/projects/mapview/). The consensus pattern was obtained with the software Tandem Repeats Finder (http://www.tandem.bu.edu/). The primer sequences for the amplification of the markers were derived from the Human Genome database (http://www.gdb.org), except for two new markers (TR89812 and TR100328) that were designed with the Primer express software. All nine markers were assayed by PCR using fluorescently end-labeled primers. PCR products were run on an ABI PRISM 310 Genetic Analyzer together with the fluorescence labeled DNA fragment size standard TAMRA (Applied Biosystems). Genotyping of two intragenic SNP located within *MLH1* exon 8 (c.655A>G) and intron 14 (c.1668-19A>G) was performed by DGGE.

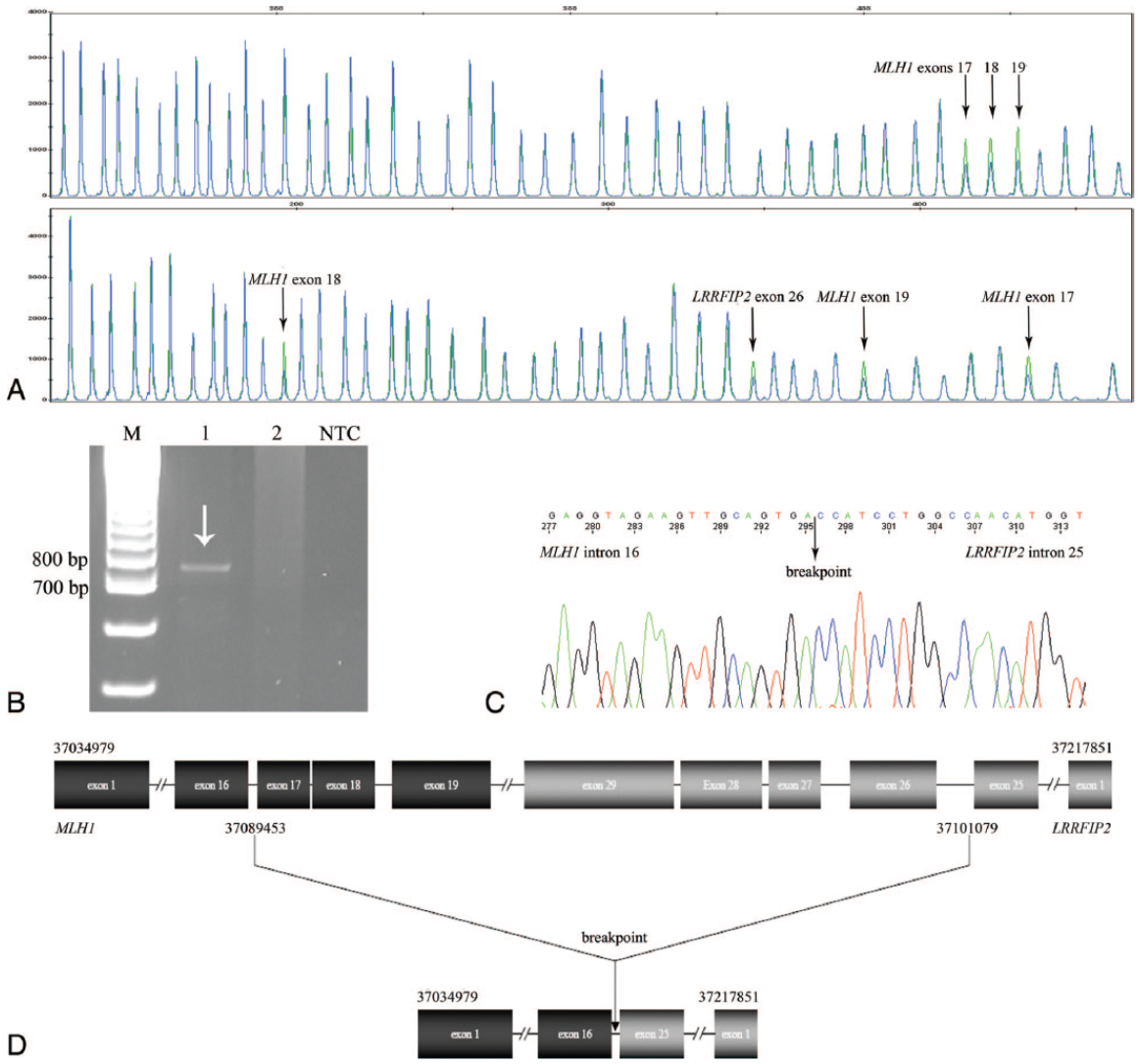

Fig. 1. Molecular characterization of the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation. A, Capillary electrophoresis pattern from one case (blue) presenting a signal reduction of approximately 50% for exons 17, 18, and 19 of *MLH1* gene (arrows) compared with a normal control (green) detected by MLPA using the SALSA MLPA P003 kit (upper panel) and the kit P248 MLH1/MSH2 (lower panel), the latter showing also a signal reduction for *LRRFIP2* exon 26 (arrows). B, Long-range PCR with primers spanning the putative breakpoints revealed a 741 bp fragment (arrow) in the cases with the *MLH1* mutation (Lane 1). Lane 2 shows a negative case. NTC is a nontemplate control and MW refers to 100 bp DNA standard. C, Sequence electrophorogram of the 741 bp PCR fragment showing the breakpoint region of the mutated allele. D, Scheme representing the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation found in all index cases, with the breakpoint downstream of *MLH1* exon 16 and upstream of *LRRFIP2* exon 25.

SNP markers were used to obtain a haplotype spanning approximately 2.6 Mb encompassing the region between TR89812 and D3S3527 microsatellite markers, where the first recombinant and/or mutational events were observed. To capture most of the genetic variation in this region and to avoid redundant SNP markers (i.e., markers in strong linkage disequilibrium), we performed Tag-SNP, namely Tagger Multimarker, using International HapMap Project CEPH (Utah residents with ancestry from northern and western Europe) population data (www.hapmap.org). We developed SNaPshot assays for 19 SNP markers by multiplexed nucleotide primer extension reaction using dye label terminators (Applied Biosystems). The primers for multiplex amplification and single base extension (Table, Supplemental Digital Content 3, http://links.lww.com/GIM/A184) were designed using the online Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). AutoDimer (www.cstl.nist.gov/strbase/NIJ/AutoDimer.htm) was used to test for potential hairpin structures and primer dimer problems. The 19 SNPs were PCR amplified in four multiplex reactions with amplicon lengths between 101 bp and 381 bp. Amplification was carried out in a 9700 Thermocycler (Applied Biosystems). After a 95°C preincubation step for 5 minutes, PCR was performed in a total of 35 cycles using the following conditions: 95°C denaturation for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C during 30 seconds, followed by 10 minutes of final extension at 72°C. The multiplex SNaPshot reaction and capillary electrophoresis were done following the manufacturer's protocol (Applied Biosystems).

Haplotype construction and estimation of mutation age

Haplotype construction was performed manually based on the genotypes obtained from index cases and family members. We estimated the age of the mutation from the variation accumulated in their ancestral haplotypes, as described by Martins et al.[17] This method takes into account both recombination (c) and mutation (μ) rates in the generation of variation. The probability of change per generation (ϵ) is given by $\epsilon = 1 - ([1 - c][1 - \mu])$, and the average of mutation and recombination events (λ) equals ϵt, where t is the number of generations. The recombination rate (c) was estimated from the physical distance between the two most distant markers (D3S1609 and D3S3522) using a conversion factor calculated in Rutgers Map Interpolator (http://compgen.rutgers.edu/old/map-interpolator/). The estimate of average mutation rate used was 7.8×10^{-4} for dinucleotides markers.[18]

RESULTS

Identification of a novel *MLH1* exonic rearrangement

Analysis of the constitutional blood-derived DNA by MLPA in the 14 index cases reported herein revealed a reduction of the peak signal for exons 17, 18, and 19 of *MLH1* of approximately 50% compared with normal controls, suggesting a heterozygous genomic deletion of these exons (Fig. 1A). Subsequent analysis with a MLPA confirmation kit revealed that exon 26 of the *LRRFIP2* gene downstream of *MLH1* was also deleted in all 14 cases (Fig. 1A). This *MLH1* c.1897-?_2271+?del (HGVS, NM_000249: initiating codon = 1) mutation is present in approximately 17% (14/84) of all Lynch syndrome families with pathogenic mutations identified at the Genetics Department of Portuguese Oncology Institute of Porto (unpublished data).

Genomic breakpoint identification

The *MLH1* c.1897-?_2271+?del (HGVS, NM_000249: initiating codon = 1) mutation was fully characterized on the nucleotide level. After long-range PCR with primers spanning the putative breakpoints, a 741 bp fragment appeared in the cases with the *MLH1* mutation (Fig. 1B). Sequence analysis of this PCR product revealed the breakpoint region in the mutated allele (Fig. 1C). All 14 probands harbored an identical 11,627 bp deletion, comprising exons 17, 18, and 19 of the *MLH1* gene and exons 26, 27, 28, and 29 of the adjacent *LRRFIP2* gene (Fig. 1D). The 5′ and 3′ breakpoints were located 280 bp downstream of *MLH1* exon 16 and 678 upstream of *LRRFIP2* exon 25, respectively. Therefore, the full description of the *MLH1* mutation is c.1896 + 280_oLRRFIP2:c.1750-678del (HGVS, NM_000249: initiating codon = 1).

Mutation-specific detection

Genomic DNA amplification by the three-primer set in the cases presenting the *MLH1* deletion resulted in two fragments of 533 bp and 551 bp from the wild-type and mutated alleles, respectively, whereas in the negative cases only the 533 bp fragment from the wild-type allele appears (Fig., Supplemental Digital Content 2, http://links.lww.com/GIM/A183). After multiplexed nucleotide primer extension reaction, the positive cases present the wild-type (G) and the mutant (C) nucleotides with the BKP1F primer and the wild-type (C) and the mutant (A) nucleotides with the BKP2-WTF and BKP2-MTF primers, respectively (Fig., Supplemental Digital Content 2, http://links.lww.com/GIM/A183). The negative cases only present the wild-type G and C nucleotides (Fig., Supplemental Digital Content 2, http://links.lww.com/GIM/A183). The SNaPshot reaction was performed on all 14 index cases and in 20 negative cases previously analyzed by direct sequencing, and all the cases were concordant.

Breakpoints sequence analysis

The genomic sequences flanking the deletion breakpoints in *MLH1* intron 16 and *LRRFIP2* intron 25 were analyzed for low-complexity DNA sequences and interspersed repeats, and one AluSx repeat and one AluSc repeat, respectively, were found at the breakpoints.

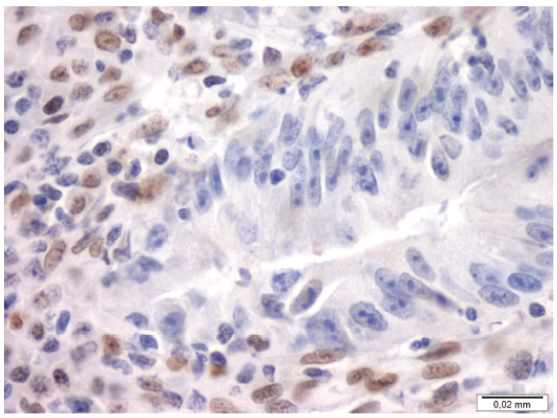

Fig. 2. Representative image of MLH1 immunostain: stromal cells show distinct nuclear immunoreactivity, whereas adenocarcinoma cells (right side) are negative.

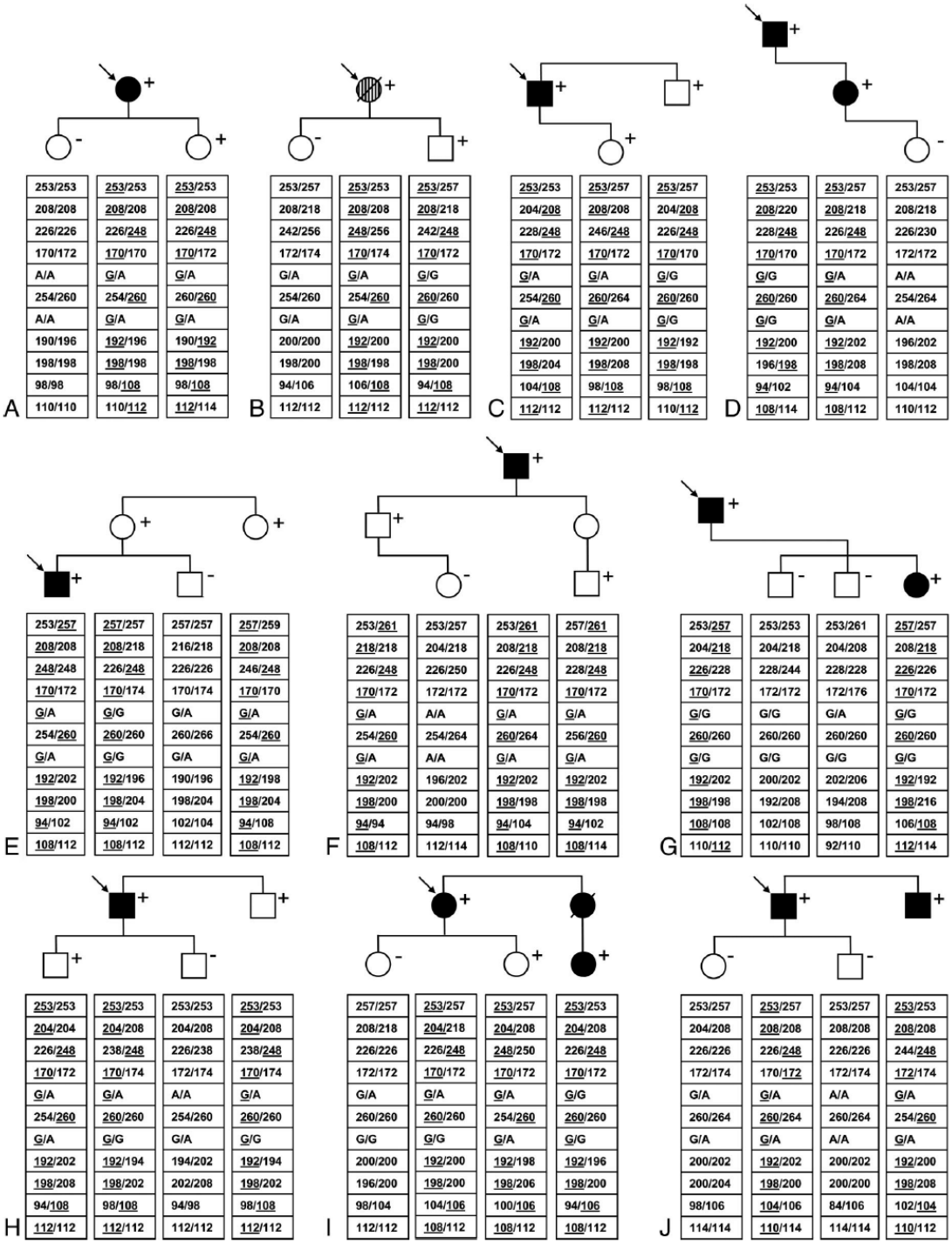

Fig. 3. Simplified pedigrees of the 10 informative families and haplotype results (A to J). The order of the microsatellite and SNP markers is D3S1609, D3S1612, D3S1561, TR89812, c.655A>G, D3S1611, c.1668-19A>G, TR100328, D3S1298, D3S3527, and D3S3522 (from top to bottom), and the alleles that segregate with the mutation are underlined. Unaffected individuals are indicated with open symbols, patients affected with colorectal cancer with black symbols, and breast cancer is represented by striped circles. The oblique line indicates that the patient is deceased. Plus and minus signals represent family members with and without the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation, respectively.

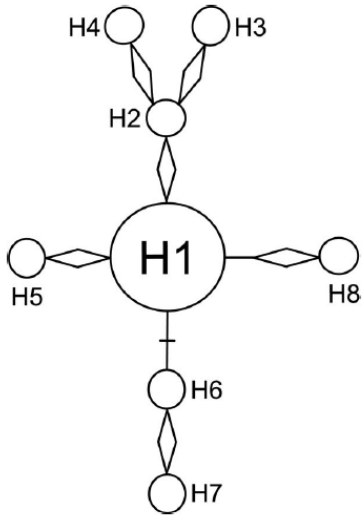

Fig. 4. Phylogenetic network showing the most parsimonious relationships among flanking short tandem repeat-based haplotypes in families carrying the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation. Circle and line sizes are proportional to the number of families and stepwise mutations, respectively, and diamonds indicate recombination events.

Table 1 Age estimation of the ancestral *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation

Haplotype[a]	Families, no.	Mutation steps, no.	Age ± δ, *y*[b]
H1: 253–208–248–170–260–192–198–108–112	3	0	283 ± 78
H2: 253–208–248–170–260–192–198–94–108	1	1	
H3: 257–**208–248–170–260–192–198**–94–108	1	2	
H4: 261–218–**248–170–260–192–198**–94–108	1	2	
H5: 257–218–226–**170–260–192–198–108–112**	1	1	
H6: 253–204–**248–170–260–192–198–108–112**	1	2	
H7: 253–204–**248–170–260–192–198**–106–108	1	3	
H8: 253–208–248–172–**260–192–198**–104–110	1	2	
	10	13	

[a]The nine microsatellite markers used were D3S1609, D3S1612, D3S1561, TR89812, D3S1611, TR100328, D3S1298, D3S3527, and D3S3522 (from left to right). The ancestral haplotype in which the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation probably occurred is indicated in bold.
[b]The recombination rate (*c*) was based on the physical distance between the two most distant markers (10848.9 kilobases; $c = 0.108672$ cM) using a conversion factor calculated in Rutgers Map Interpolator. The estimated probability of mutation per generation and per haplotype was 0.00702 (as nine dinucleotide short tandem repeats were studied).

MMR immunohistochemical and microsatellite instability analyses

All five carcinomas and the tubulovillous adenoma studied by immunohistochemistry showed absence of MLH1 expression (Fig. 2). Additionally, both the carcinoma and the tubulovillous adenoma of the two index cases with Bethesda criteria showed high microsatellite instability (all five markers presented instability).

Ancestral STR-based haplotypes and age estimate

Eight different haplotypes were phased for 10 of the 14 families. The results of the haplotype analyses for the 10 informative families are shown in Figure 3, and the most parsimonious relationships among flanking haplotypes are presented as a phylogenetic network in Figure 4. The probability of mutation versus recombination was evaluated considering the number of stepwise mutations required and intermediate haplotypes observed. In the 10 informative families, SNP haplotypes were constructed to establish whether a specific microsatellite was different from the consensus because of a recombination event rather than a mutation (Fig., Supplemental Digital Content 4, http://links.lww.com/GIM/A185).

Haplotype analysis of the 10 informative families revealed a conserved region of approximately 1 Mb. Based on the mutation and recombination events observed in microsatellite haplotypes and assuming a generation time of 25 years, the age estimate for the *MLH1* mutation c.1896 + 280_oLRRFIP2:c.1750-678del (HGVS, NM_000249: initiating codon = 1) is 283 ± 78 years (Table 1).

The geographic origins of the *MLH1* mutation positive families are shown in Figure 5, all being originated from the district of Porto, Portugal, but away from the most densely populated areas (the city of Porto or surrounding cities). The remaining 70 Lynch families identified at our institution have a much disperse geographic origin from the entire North and Center of Portugal.

Clinicopathologic associations

CRC was the most frequent malignancy (73%) observed in the families presenting the *MLH1* exonic rearrangement reported herein, followed by stomach (7%) and endometrial cancer (5%). The median age of diagnosis of CRC was 44 years. Seven patients presented metachronous and one patient presented synchronous colorectal carcinomas. The histomorphological study of all five colorectal carcinomas that could be evaluated were adenocarcinomas (one poorly and four moderately to well differentiated), and mucinous production was observed in three of the carcinomas. Three of the five carcinomas were located in the right colon.

DISCUSSION

The novel *MLH1* mutation c.1896 + 280_oLRRFIP2:c.1750-678del that we report herein was identified in 14 Portuguese Lynch syndrome families, representing approximately 17% (14/84) of all deleterious MMR mutations and approximately 41% (14/34) of the *MLH1* mutations detected at the Genetics Department of Portuguese Oncology Institute of Porto (unpublished data). All the families presenting this mutation have their origin in a small geographic area in the north of Portugal, comprising several counties in the periphery of the Porto district. At least 20% (17/84) of all Lynch syndrome families with a pathogenic mutation identified at our institution

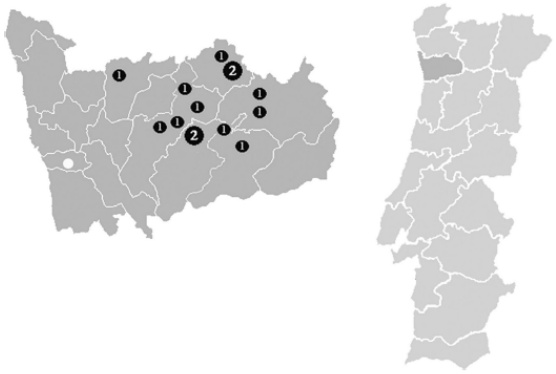

Fig. 5. Geographic origin of the families with the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del germline mutation in Portugal. Black circles and the number within represent the families and its frequency. All families are originated from a small region of the district of Porto (shadowed region at the right) but not from the city of Porto itself, which is located as indicated by the white circle.

have their origin from other regions of Portugal, and none of the 14 families with this *MLH1* rearrangement originated from the most densely populated areas of Porto district. Furthermore, none of the previous publications on Portuguese Lynch families mostly from South Portugal reported this exonic rearrangement.[19,20] These data indicate that our finding of this mutation in 14 families from the Porto district is not explained by referral bias. In other countries, the deletion of exons 17–19 of the *MLH1* gene has been reported in one Lynch syndrome family from Taiwan[21] and another from France.[22] However, none of these studies described the genomic breakpoints or deletion of *LRRFIP2* exon 26 (detectable by the MLPA P248 MLH1/MSH2 kit), so one can assume that the genomic rearrangement reported herein is novel. In fact, it has recently been shown that the breakpoints of the *MLH1* rearrangement reported in the Lynch syndrome family from Taiwan[21] are different from the ones we describe in this study (Dr. Ling-Ling Hsieh, Chang Gung University, Taiwan, personal communication, 2010).

Haplotype analysis by microsatellite and SNP markers flanking the *MLH1* gene in the 10 informative families revealed a conserved region of approximately 1 Mb, indicating that these families indeed share a common ancestor. Based on the mutation and recombination events observed in microsatellite haplotypes and assuming a generation time of 25 years, the origin of this mutation could be traced back to the beginning of the 18th century. This relatively young age is in agreement with the confined geographic origin of the 14 families bearing the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation. Furthermore, the same exact breakpoint was identified in each of the families, which is also a strong indicator of a common origin. The available data, therefore, indicates that this is a novel exonic rearrangement involving deletion of the last three exons of *MLH1* and the last four exons of the contiguous *LRRFIP2* gene, being a frequent founder mutation in Lynch families originated from the Porto district in North Portugal.

Several studies have shown that genomic deletions and duplications in the *MSH2* or *MLH1* genes are a frequent cause of Lynch syndrome.[7,9,23–27] Wijnen et al.[25] reported a frequency of 6.5% of *MSH2* deletions in Lynch families from a Dutch population. More recently, large MMR gene rearrangements have been reported in 11–15% of Lynch syndrome families in France,[26] The Netherlands,[7] and Germany,[27] in all instances most commonly in *MSH2*. The higher frequency of large genomic rearrangements in *MSH2* is presumably due to a higher Alu density (34.2% on average).[26] Alu repeats are short interspersed elements whose transposition has been repeatedly implicated in genetic variability and heritable disorders, including Lynch syndrome and hereditary breast/ovarian cancer.[27,28] In the *MLH1* gene, Alu-mediated exonic deletions have previously been reported mainly associated with a founder effect, as the deletion of exon[16] represents approximately 50% of all *MLH1* mutations in the Finnish population.[23] This Finnish founder mutation, a 3.5 kb *MLH1* deletion, resulted from a recombination event between two Alu repeats located in introns 15 and 16.[23] Mauillon et al.[24] also observed a deletion of exons 13–16 of the *MLH1* gene, caused by a recombination event between two Alu repeats located in introns 12 and 16. As we found Alu repeats around the breakpoints of both *MLH1* intron 16 and *LRRFIP2* intron 25, Alu-mediated homologous recombination might also have been involved in the origin of the Portuguese founder exonic rearrangement that we report in this study.

The MLH1 protein forms a heterodimer with MLH3, PMS2, or PMS1 and recruits other DNA repair proteins to the MMR complex for the excision and repair of DNA.[29] The *MLH1* exons deleted in the rearrangement that we describe (exons 17–19) code for the MLH3, PMS2, or PMS1-binding domain. The carcinoma and the adenoma analyzed for microsatellite status presented high instability, which is indicative of MMR deficiency. Moreover, all the tumors evaluated for MMR immunoexpression lacked MLH1 protein expression, demonstrating that this large rearrangement leads to loss of protein. On the other hand, this rearrangement affects also the adjacent *LRRFIP2* gene, causing deletion of the last four exons (26–29). *LRRFIP2* was recently identified as a modulator of the Wingless-type mouse mammary tumor virus integration site family (Wnt) signaling pathway.[30] LRRFIP2 interacts with disheveled (Dvl) to increase the cellular abundance of β-catenin and activates LEF/TCF-dependent gene transcription of Wnt target genes. It presents a coiled-coil domain at its carboxyl terminus and a serine-rich region at the amino terminus.[31] Liu et al.[30] analyzed the molecular function of LRRFIP2 with a series of deleted mutants and observed that the activity of LRRFIP2 was severely reduced after truncation of either the carboxyl terminus or the amino terminus, indicating that both domains are required for its function. These authors also demonstrated that a mutant form of *LRRFIP2* containing only the amino terminus acts as a dominant negative form and abolishes the activities of both LRRFIP2 and Dvl.[30] The *LRRFIP2* exons deleted in the rearrangement we here present code for the LRRFIP2 coiled-coil domain at its carboxyl terminus. If this rearrangement results in a truncated protein that exerts a dominant negative effect on the wild-type LRRFIP2, this would result in decreased β-catenin levels, which is the opposite of what is found in most CRCs. Further studies are warranted to clarify the role of this *LRRFIP2* germline mutation, if any, in the context of Lynch syndrome. On the other hand, our 14 Lynch syndrome families show typical features of this disease, such as predominance of right colon carcinomas, early onset, high microsatellite instability, and lack of MLH1 expression in tumor tissue, thereby indicating that the relevant genetic defect underlying Lynch syndrome in these families is the inactivation of *MLH1* gene through this large exonic rearrangement.

Because of the high frequency of the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation in our population, we

developed a mutation-specific assay to screen for this mutation quickly and inexpensively, which involves a three-primer PCR-specific amplification assay followed by a single-nucleotide primer extension reaction. Although the MLPA technique allows identification of the deletion of *MLH1* exons 17, 18, and 19 while screening for all *MLH1* and *MSH2* exonic rearrangements, it requires another methodology to fully characterize this specific mutation. Using our approach, the breakpoint region of the *MLH1* and *LRRFIP2* deletion is interrogated in a single, multiplex reaction, providing two independent assessments of the mutation in question. This mutation-specific assay is a faster and less expensive method for the detection of this rearrangement as it involves only a standard PCR followed by a SNaPshot reaction. In addition, this method is highly flexible, and more mutations can be added to the multiplex reaction.

In conclusion, we have identified a novel *MLH1* exonic rearrangement that is a common founder mutation in Lynch syndrome families originated from the Porto district in North Portugal. This rearrangement corresponds to a large deletion involving *MLH1* exons 17–19 and *LRRFIP2* exons 26–29, which has presumably resulted from homologous recombination between two Alu sequences present in introns 16 and 25 of the genes *MLH1* and *LRRFIP2*, respectively. The high proportion of the *MLH1* c.1896 + 280_oLRRFIP2:c.1750-678del mutation indicates that screening for this rearrangement as a first step may be cost-effective during genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the Porto district.

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REFERENCES

1. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *N Engl J Med* 2003;348:919–932.
2. Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999; 116:1453–1456.
3. Umar A, Risinger JI, Hawk ET, Barrett JC. Testing guidelines for hereditary non polyposis colorectal cancer. *Nat Rev Cancer* 2004;4:153–158.
4. Baudhuin LM, Burgart LJ, Leontovich O, Thibodeau SN. Use of microsatellite instability and immunohistochemistry testing for the identification of individuals at risk for Lynch syndrome. *Fam Cancer* 2005;4:255–265.
5. Vasen HF, Möslein G, Alonso A, et al. Guidelines for the clinical management of Lynch syndrome (hereditary non-polyposis cancer). *J Med Genet* 2007;44:353–362.
6. Lagerstedt Robinson K, Liu T, et al. A. Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. *J Natl Cancer Inst* 2007;99:291–299.
7. Gille JJ, Hogervorst FB, Pals G, et al. Genomic deletions of MSH2 and MLH1 in colorectal cancer families detected by a novel mutation detection approach. *Br J Cancer* 2002;87:892–897.
8. Stenson PD, Mort M, Ball EV, et al. The Human Gene Mutation Database: 2008 update. *Genome Med* 2009;1:13.
9. Chan TL, Yuen ST, Ho JW, et al. A novel germline 1.8-kb deletion of hMLH1 mimicking alternative splicing: a founder mutation in the Chinese population. *Oncogene* 2001;20:2976–2981.
10. Wagner A, Barrows A, Wijnen JT, et al. Molecular analysis of hereditary nonpolyposis colorectal cancer in the United States: high mutation detection rate among clinically selected families and characterization of an American founder genomic deletion of the MSH2 gene. *Am J Hum Genet* 2003;72: 1088–1100.
11. Caluseriu O, Di Gregorio C, Lucci-Cordisco E, et al. A founder MLH1 mutation in families from the districts of Modena and Reggio-Emilia in northern Italy with hereditary non-polyposis colorectal cancer associated with protein elongation and instability. *J Med Genet* 2004;41:34.
12. Sun S, Greenwood CM, Thiffault I, Hamel N, Chong G, Foulkes WD. The HNPCC associated MSH2*1906G–>C founder mutation probably originated between 1440 CE and 1715 CE in the Ashkenazi Jewish population. *J Med Genet* 2005;42:766–768.
13. Müllenbach R, Lagoda PJ, Welter C. An efficient salt-chloroform extraction of DNA from blood and tissues. *Trends Genet* 1989;5:391.
14. Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. *Cancer Res* 1997;57:4749–4756.
15. Pinheiro M, Ahlquist T, Danielsen SA, et al. Colorectal carcinomas with microsatellite instability display a different pattern of target gene mutations according to large bowel site of origin. *BMC Cancer* 2010;10:587.
16. Wu Y, Berends MJ, Mensink RG, et al. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. *Am J Hum Genet* 1999;65:1291–1298.
17. Martins S, Calafell F, Gaspar C, et al. Asian origin for the worldwide-spread mutational event in Machado-Joseph disease. *Arch Neurol* 2007;64:1502–1508.
18. Gyapay G, Morissette J, Vignal A, et al. The 1993-94 Généthon human genetic linkage map. *Nat Genet* 1994;7:246–339.
19. Sousa R, Lage P, Ferreira S, et al. Need of new clinical criteria for the identification of genetic Lynch syndrome. *Acta Med Port* 2007;20:535–542.
20. Ferreira S, Lage P, Sousa R, et al. Familial colorectal cancer type X: clinical, pathological and molecular characterization. *Acta Med Port* 2009;22:207–214.
21. Tang R, Hsiung C, Wang JY, et al. Germ line MLH1 and MSH2 mutations in Taiwanese Lynch syndrome families: characterization of a founder genomic mutation in the MLH1 gene. *Clin Genet* 2009;75:334–345.
22. Rouleau E, Lefol C, Bourdon V, et al. Quantitative PCR high-resolution melting (qPCR-HRM) curve analysis, a new approach to simultaneously screen point mutations and large rearrangements: application to MLH1 germline mutations in Lynch syndrome. *Hum Mutat* 2009;30:867–875.
23. Nyström-Lahti M, Kristo P, Nicolaides NC, et al. Founding mutations and Alu-mediated recombination in hereditary colon cancer. *Nat Med* 1995;1: 1203–1206.
24. Mauillon JL, Michel P, Limacher JM, et al. Identification of novel germline hMLH1 mutations including a 22 kb Alu-mediated deletion in patients with familial colorectal cancer. *Cancer Res* 1996;56:5728–5733.
25. Wijnen J, van der Klift H, Vasen H, et al. MSH2 genomic deletions are a frequent cause of HNPCC. *Nat Genet* 1998;20:326–328.
26. Charbonnier F, Olschwang S, Wang Q, et al. MSH2 in contrast to MLH1 and MSH6 is frequently inactivated by exonic and promoter rearrangements in hereditary nonpolyposis colorectal cancer. *Cancer Res* 2002; 62:848–853.
27. Wang Y, Friedl W, Lamberti C, et al. E. Hereditary nonpolyposis colorectal cancer: frequent occurrence of large genomic deletions in MSH2 and MLH1 genes. *Int J Cancer* 2003;103:636–641.
28. Peixoto A, Santos C, Rocha P, et al. The c. 156_157insAlu BRCA2 rearrangement accounts for more than one-fourth of deleterious BRCA mutations in northern/central Portugal. *Breast Cancer Res Treat* 2009;114:31–38.
29. Mitchell RJ, Farrington SM, Dunlop MG, Campbell H. Mismatch repair genes hMLH1 and hMSH2 and colorectal cancer: a HuGE review. *Am J Epidemiol* 2002;156:885–902.
30. Liu J, Bang AG, Kintner C, et al. Identification of the Wnt signaling activator leucine-rich repeat in Flightless interaction protein 2 by a genome-wide functional analysis. *Proc Natl Acad Sci USA* 2005;102:1927–1932.
31. Fong KS, de Couet HG. Novel proteins interacting with the leucine-rich repeat domain of human flightless-I identified by the yeast two-hybrid system. *Genomics* 1999;58:146–157.

PAPER II

Manuela Pinheiro, Carla Pinto, Ana Peixoto, Isabel Veiga, Bárbara Mesquita, Rui Henrique, Paula Lopes, Olga Sousa, Maria Fragoso, Luís Moreira Dias, Manuela Baptista, Carla Marinho, Elizabeth Mangold, Carlos Vaccaro, Gareth D. Evans, Susan Farrington, Malcolm G. Dunlop, Manuel R. Teixeira.

"The MSH2 c.388_389del mutation shows a founder effect in Portuguese Lynch syndrome families"

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Short Report

The *MSH2* c.388_389del mutation shows a founder effect in Portuguese Lynch syndrome families

Pinheiro M, Pinto C, Peixoto A, Veiga I, Mesquita B, Henrique R, Lopes P, Sousa O, Fragoso M, Dias LM, Baptista M, Marinho C, Mangold E, Vaccaro C, Evans DG, Farrington S, Dunlop MG, Teixeira MR. The *MSH2* c.388_389del mutation shows a founder effect in Portuguese Lynch syndrome families.
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The *MSH2* c.388_389del mutation has occasionally been described in Lynch families worldwide. At the Portuguese Oncology Institute in Porto, Portugal, we have identified 16 seemingly unrelated families with this germline mutation. To evaluate if this alteration is a founder or a recurrent mutation we performed haplotype analysis in the 16 Portuguese index cases and 55 relatives, as well as in four index cases and 13 relatives reported from Germany, Scotland, England, and Argentina. In the Portuguese families we observed a shared haplotype of approximately 10 Mb and all were originated from the north of Portugal. These results suggest that this alteration is a founder mutation in Portugal with a relatively recent origin. In the reported families outside Portugal with this mutation different haplotype backgrounds were observed, supporting the hypothesis that it occurred *de novo* on multiple occasions. We also conclude that the high proportion of families with the *MSH2* c.388_389del mutation indicates that screening for this alteration as a first step may be cost-effective in the genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal.

Conflict of interest

The authors declare no conflict of interest.

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Key words: founder mutation – Lynch syndrome – *MSH2* – recurrent mutation

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Although Lynch syndrome is characterized by marked genetic heterogeneity, a few specific mutations are observed at high frequencies in well-defined populations or ethnic groups, due to founder effects (1, 2). Recently, we identified the founder mutation *MLH1* c.1896+280_oLRRFIP2:c.1750-678del that represents 17% of all deleterious MMR mutations in Portuguese Lynch syndrome families (3). The presence of common founder mutations can greatly facilitate Lynch syndrome molecular diagnosis by allowing targeted mutational analysis to specific gene regions as the first step of the genetic testing strategy.

During genetic testing for Lynch syndrome at the Portuguese Oncology Institute, Porto (IPO-Porto), we identified 15 novel, apparently unrelated, families with the *MSH2* c.388_389del mutation. This germline mutation was first described in one Lynch family in Germany and since then it has been reported in single Lynch syndrome families from Scotland, England, and Argentina (4–8). This mutation was also incidentally identified by us in a patient with a rectal tumor and no relevant family history of cancer (9). During the course of this study, this mutation has also been described in four Lynch syndrome families from France (10) and in one family from the North American and Australian Colorectal Cancer Family Registry (Colon CFR) (11). Given the high frequency of the *MSH2* c.388_389del mutation among our series of 103 Lynch syndrome families with deleterious mutations, we aimed to determine the geographical distribution and ancestral origin of this genetic alteration in Portugal and to compare it with those reported elsewhere.

Materials and methods

Patients, samples, and DNA extraction

This study includes 15 novel Lynch syndrome families from a total series of 103 with *MLH1, MSH2, MSH6,* or *PMS2* deleterious germline mutations identified at IPO-Porto (data not shown). After written informed consent, DNA was isolated from peripheral blood samples from the 15 probands and subsequently from 55 family members. The geographic origin of these families was inferred from the birthplace of the oldest carrier or of the oldest affected family member most likely to be a carrier.

For the purpose of haplotype studies, we included the patient previously described by our group (9), as well as all four Lynch syndrome families that had been described outside Portugal at the time with the *MSH2* c.388_389del mutation. These included one index case from Germany (4, 5), one index case and three relatives each from Scotland (6) and England (7), and one index case and seven relatives from Argentina (8).

Genetic testing strategy

In eight of the 10 families that fulfilled the Amsterdam criteria and in one of the five cases presenting the Bethesda criteria (for whom a tumor sample was not available), germline mutation screening was performed directly in a blood sample of the index case. In two families presenting the Amsterdam criteria and in one presenting the Bethesda criteria, MLH1/MSH2/MSH6/PMS2 immunoexpression was initially assessed, whereas in the remaining three families with Bethesda criteria, microsatellite instability analysis was performed before germline mutation analysis, both as previously described (9). In addition, MSH2/MSH6 immunoexpression was subsequently assessed in six tumors from six families with Amsterdam criteria (five index cases and one affected relative from another family) and in one index case from a family with Bethesda criteria. Germline mutation analysis was performed by denaturing gradient gel electrophoresis (DGGE) and direct sequencing (3). The 55 family members were screened for the *MSH2* c.388_389del mutation by direct sequencing.

Microsatellite and single nucleotide polymorphisms (SNP) typing and haplotype construction

Probands and their relatives were genotyped for the 10 microsatellite markers indicated in Table 2. All 10 markers were assayed by PCR using fluorescently end-labeled primers and capillary electrophoresis was performed on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA). Genotyping of three intragenic SNP located within *MSH2* intron 9 (c.1511-9T>A), intron 10 (c.1661+12G>A), and intron 12 (c.2006-6T>C) was performed by DGGE and SNaPshot assays (Applied Biosystems) were used for additional 11 SNP markers (located between D2S391 and D2S378). Haplotype construction was performed manually.

Results

Lynch syndrome families' characterization

The *MSH2* c.388_389del (HGVS, NM_000251: ATG = 1) mutation (Fig. 1) was identified in 16 (15 novel) from a total of 103 Lynch syndrome families with deleterious germline mutations, accounting for about 16% of all positive families in our series

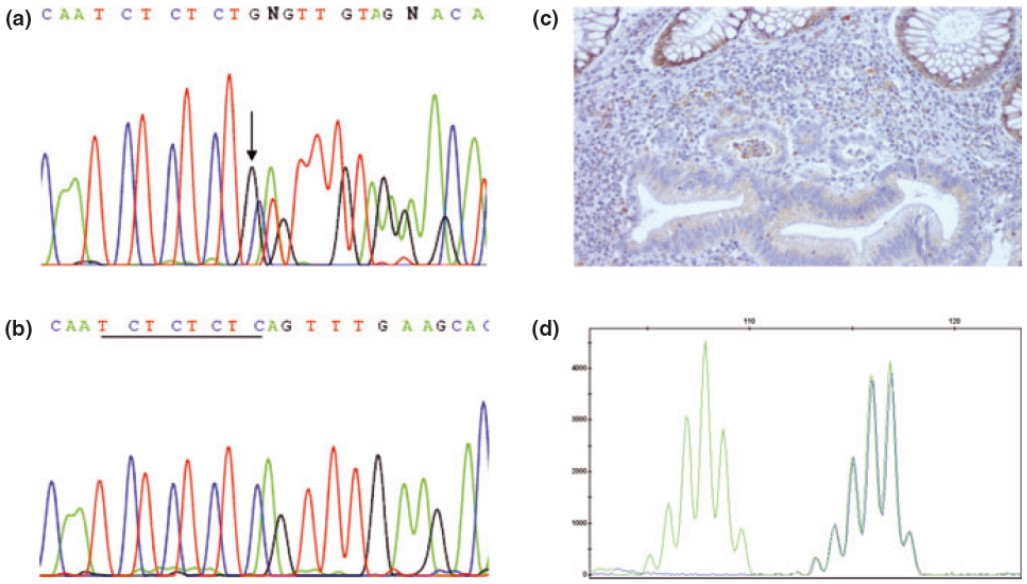

Fig. 1. (**a**) Sequence electropherogram of a patient with the *MSH2* c.388_389del mutation (indicated by an arrow). (**b**) Sequence electropherogram of one wild type case with the short repeat motif (TCTCTCTC) immediately upstream of the deletion underlined. (**c**) Representative image of MSH2 immunostain: normal glands and stromal cells show nuclear immunoreactivity, whereas adenocarcinoma cells are negative. (**d**) Fragment analysis electropherogram showing microsatellite instability in BAT26 microsatellite marker in one colorectal tumor DNA (green) paired with the corresponding normal DNA (blue).

Table 1. Clinical criteria, index tumor type, microsatellite instability and immunohistochemical results for the 15 novel Lynch syndrome families presenting the *MSH2* c.388_389del mutation

Family	Criteria	Index tumor type (age at diagnosis)	CRC location	MSI	IHC
1	Amsterdam	CRC (44)	Right colon	n.a.	MSH2/MSH6 loss
2	Amsterdam	OC (38); EC (42); CRC (52)	Right colon	n.a.	MSH2/MSH6 loss
3	Amsterdam	CRC (46, 54)	Left and right colon	n.a.	MSH2/MSH6 loss
4	Amsterdam	CRC (30)	Left colon	n.a.	MSH2/MSH6 loss
5	Amsterdam	Polyps (45)	Un	n.a.	n.a.
6	Bethesda	GC, 2 CRC (43, 47 and 49)	Left and right colon	MSI-H	MSH2/MSH6 loss
7	Amsterdam	Un	Un	n.a.	MSH2/MSH6 loss[a]
8	Bethesda	CRC (44)	Left colon	MSI-H	n.a.
9	Amsterdam	CRC (45)	Un	n.a.	n.a.
10	Amsterdam	CRC and EC (46)	Right colon	n.a.	MSH2/MSH6 loss
11	Bethesda	CRC (41)	Right colon	MSI-H	n.a.
12	Bethesda	CRC (29)	Left colon	n.a.	n.a.
13	Amsterdam	Adenoma, high grade dysplasia (36)	Left colon	n.a.	MSH2/MSH6 loss
14	Amsterdam	CRC, EC (55,56)	Right colon	n.a.	MSH2/MSH6 loss
15	Bethesda	PC (64), ST (70), CRC (73), Un (84)	Right colon	n.a.	MSH2/MSH6 loss

CRC, colorectal cancer; EC, endometrium cancer; GC, gastric cancer; IHC, Immunohistochemical analysis; MSI, Microsatellite instability; MSI-H, High microsatellite instability; n.a., not analyzed; OC, ovarian cancer; PC, prostate cancer; ST, soft tissue cancer; Un, unknown

[a]MMR immunohistochemical analysis performed in one adenoma from a relative of the index case.

(unpublished data). In addition, 29 relatives of the 55 analyzed presented the *MSH2* c.388_389del mutation.

All eight carcinomas and two tubulovillous adenomas studied by immunohistochemistry showed absence of MSH2 and MSH6 expression (Fig. 1; Table 1). In addition, the three carcinomas in probands with Bethesda criteria showed high microsatellite instability (MSI-H) (Fig. 1; Table 1).

Haplotype analysis

Microsatellite haplotypes for all markers were phased for seven out of the 15 Portuguese Lynch syndrome families analyzed and two different haplotypes were observed. Six families presented the same haplotype and one family only differed for the D2S391 microsatellite marker, revealing a conserved region of approximately 10 Mb (Table 2). All mutation carriers of

Table 2. Microsatellite and single nucleotide polymorphism marker haplotypes of the Portuguese, German, Scottish, English and Argentinean Lynch syndrome families presenting the *MSH2* c.388_389del mutation

		Portuguese families[b]															Families outside Portugal[b]			
		Phased haplotype							Non informative families											
Markers	Position (Mb)[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16[c]	Germany	Scotland	England	Argentina
tel																					
D2S391	1.22	142	140	140	140	140	140	140	140/146	140/142	140/146	140/148	140	140/146	140/142	140/146	140	140	**144**	**144**	140
D2S2227	0.36	117	117	117	117	117	117	117	117	115/117	117/125	117	117	117	117	115/117	117	117	117	117	117
Clen33	0.22	160	160	160	160	160	160	160	158/160	160	158/160	160/164	160	160/162	160/164	158/160	160/162	160/162	**158**	**158**	160
c.1511-9T>A	Intragenic	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
c.1661+12G>A	Intragenic	G	G	G	G	G	G	G	G	G	G	G	G	G	G/A	G	G	G	G	G	G/A
c.2006-6T>C	Intragenic	T	T	T	T	T	T	T	T	T	T	T	T	T	T/C	T	T	T	T	T	T/C
MSH2																					
Clen30	0.17	272	272	272	272	272	272	272	272	272	266/272	270/272	272	272	272	268/272	270/272	**270/274**	**266**	**266**	272/274
Clen29	1.63	146	146	146	146	146	146	146	146/172	146/172	146	146/174	**164/172**	146/172	146/172	146/172	146/172	146/172	**172**	**172**	**164**
D2S2156	3.63	123	123	123	123	123	123	123	123	123	123	123	**121/125**	121/123	119/123	123	123	123	123	123	123
D2S123	3.66	209	209	209	209	209	209	209	209	209/225	209	207/209	209/227	209/225	209/213	209	209/223	209	**225**	**225**	209
Clen 43	4.56	177	177	177	177	177	177	177	169/177	177	163/177	173/177	169/177	177	177	163/177	177/179	177	**163**	**163**	**173**
Clen 44	7.50	120	120	120	120	120	120	120	**118/132**	120	120	112/120	116/120	120	120	120/124	112/120	**112/126**	**118**	120	120
D2S378	9.67	211	211	211	211	211	211	211	201/211	201/211	211/215	211/217	**201/215**	211/215	211/215	211/215	211	211/215	**203**	**209**	**203**
cen																					

[a]Physical distances of the genetic markers were derived from the National Center for Biotechnology Information (NCBI) Map Viewer.
[b]The alleles that differ from the most frequent haplotype are indicated in bold.
[c]Patient previously described by our group to present the *MSH2* c.388_389del mutation (see Ref. 3).

Fig. 2. (**a**) Geographic origin of the families identified with the *MSH2* c.388_389del mutation in Portugal. All families originated from the north of Portugal (shadowed region). (**b**) Larger-scale map of the north of Portugal shadowed region with the white circles and the number within representing the families and its frequency.

unphased haplotypes harbored alleles consistent (indicated in bold in Table 2) with one or both haplotypes, with the exception of families 8 and 12. The previously reported Portuguese patient with the *MSH2* c.388_389del mutation also presented an unphased haplotype consistent with the most frequent haplotype. The geographic origins of the 16 known Portuguese families carrying this germline mutation are shown in Fig. 2, all being originated from the north of Portugal.

Regarding the previously reported families with the *MSH2* c.388_389del mutation living in other countries, we could only phase a complete haplotype for the families from England and Scotland. They presented different haplotypes from the Portuguese, German, and Argentinean families (Table 2). These two Lynch syndrome families from the United Kingdom shared a region of approximately 5.8 Mb, only differing for markers Clen44 and D2S378 (Table 2). Although we could not phase a complete haplotype for the German and Argentinean families, they appear to share between them and with the most frequent Portuguese haplotype a region of approximately 1.3 Mb encompassing the most telomeric markers (from the *MSH2* gene to the D2S391 microsatellite marker), differing for the remaining markers (from *MSH2* gene to D2S378).

SNP haplotypes were also constructed in the seven Portuguese microsatellite haplotype informative families and in the four families from other countries (Table 3). Although we could not phase completely a SNP haplotype for the seven Portuguese informative families, we observed two different haplotypes, one in the six families that presented the same microsatellite haplotype and the other in the case that differed for the D2S391 microsatellite marker. In the Lynch syndrome families from other countries, the SNP haplotype observed was also in accordance with the results obtained for the microsatellite markers.

Discussion

We detected the *MSH2* c.388_389del mutation in a total of 16 of 103 (16%) families with deleterious MMR germline mutations so far identified at IPO-Porto. The Lynch syndrome families we report here showed typical features of this disease, such as early onset, MSI-H instability and lack of MSH2 expression in tumor tissue, as described for the previously reported cases.

Owing to the high frequency of the *MSH2* c.388_389del mutation in our series and the various reports of its presence in other countries, we aimed to determine if this is a founder or a recurrent mutation. The seven informative Portuguese families with the *MSH2* c.388_389del mutation shared an identical microsatellite haplotype covering a chromosomal region of approximately 10 Mb. All the 16 known Portuguese families are originated from the north of Portugal and, as far as we could go on genealogical studies, these families are not related. In addition, none of the previous publications on Portuguese Lynch families mostly from central/south Portugal reported the *MSH2* c.388_389del mutation (12–17), so the geographical distribution we observe is not likely to be explained by a referral bias to our institution. The shared haplotype length and the limited geographical dispersion in Portugal indicate that the *MSH2* c.388_389del alteration is a founder mutation in Portugal with a relatively recent origin.

The Lynch syndrome families with the *MSH2* c.388_389del mutation living outside Portugal that we studied showed no clear evidence for a common ancestry nor do they report any Portuguese ancestry. A clearly distinct microsatellite haplotype was identified for the families from England and Scotland, whereas the German and Argentinean families appeared to share between them the most frequent Portuguese haplotype in a region of approximately 1.3 Mb. Theoretically, recombination or mutation events could account for haplotype divergence from a single ancestor, but given the different haplotypes observed in these worldwide dispersed families, the simplest explanation is that this mutation occurred several times *de novo*. SNP markers

Table 3. Single nucleotide polymorphism marker haplotypes observed in the Lynch syndrome families presenting the *MSH2* c.388_389del mutation from Portugal (with a microsatellite informative haplotype), Germany, Scotland, England and Argentina

		Portuguese families							Families outside Portugal			
SNP[a]	Position (Mb)	1	2	3	4	5	6	7	Bonn	Edinburgh	Manchester	Buenos Aires
D2S391												
rs281493	1.22	T	G	G	G	G	G	G	G	G/T	G	G
rs935376	0.59	C	C	C	C	C	C	C	C	T	T	C/T
rs3923559	0.02	A	A	A	A	A	A	A	G/A	A	A	G/A
MSH2												
rs4589808	1.44	C	C	C	C	C	C	C	C/T	T	T	C/T
rs12998574	2.49	T	T	T	T	T	T	T	C	T	T	T
rs1401178	3.26	T	T	T	T	T	T	T	T	C	C	C
rs7595930	4.41	C	C	C	C	C	C	C	C/T	C	C	C
rs7601345	5.23	T	T	C/T	T	T	C/T	T	C	T	T	C
rs7597885	6.22	A	A	G/A	A	A	G/A	A	G/A	G	G	G/A
rs2920880	7.67	A	A	A	A	A	A	A	G/A	G	A	G
rs891881	8.65	C	C	C	C	C	C	C/T	C	T	T	T
D2S378												

SNP, single nucleotide polymorphism.
[a]SNP selection was performed using Tag-SNP, namely Tagger Multimarker, with the International HapMap Project CEPH (Utah residents with ancestry from northern and western Europe) population data (www.hapmap.org).

have much lower mutation rates than microsatellite markers, thus much less genetic divergence is expected to have occurred at the SNP markers in a more recent mutation (18, 19). The observed SNP haplotypes suggests that the *MSH2* c.388_389del mutation occurred in the background of different haplotypes worldwide and that the two Portuguese haplotypes most likely arose from a single common ancestor. Considering that the c.388_389del mutation appeared independently several times in the human genome, this region may be a mutational hotspot within the *MSH2* gene, as in those caused by short repeats and/or palindromes (20). In fact, a short repeat motif (TCTCTCTC) exists immediately upstream of the deletion (Fig. 1) and we hypothesize that it may be operative in the recurrent *de novo* origin of this mutation.

In conclusion, our results support the hypothesis that the *MSH2* c.388_389del mutation has occurred *de novo* on multiple occasions in different haplotype backgrounds but shows a founder effect in Portugal. The *MSH2* c.388_389del mutation reported herein constitutes 16% of our Lynch syndrome families with identified germline mutations. This indicates that screening for this *MSH2* mutation as a first step, together with the previously reported Portuguese founder mutation *MLH1* c.1896+280_oLRRFIP2:c.1750-678del (present in additional 17% of our Lynch syndrome families; see Ref. (3), may increase the cost-effectiveness of genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal.

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References

1. Wagner A, Barrows A, Wijnen JT et al. Molecular analysis of hereditary nonpolyposis colorectal cancer in the United States: high mutation detection rate among clinically selected families and characterization of an American founder genomic deletion of the MSH2 gene. Am J Hum Genet 2003: 72 (5): 1088–1100.
2. Sun S, Greenwood CM, Thiffault I, Hamel N, Chong G, Foulkes WD. The HNPCC associated MSH2*1906G→C founder mutation probably originated between 1440 CE and 1715 CE in the Ashkenazi Jewish population. J Med Genet 2005: 42 (10): 766–768.
3. Pinheiro M, Pinto C, Peixoto A et al. A novel exonic rearrangement affecting MLH1 and the contiguous LRRFIP2 is a founder mutation in Portuguese Lynch syndrome families. Genet Med 2011: 13 (10): 895–902.
4. Mangold E, Pagenstecher C, Friedl W et al. Tumours from MSH2 mutation carriers show loss of MSH2 expression but many tumours from MLH1 mutation carriers exhibit weak positive MLH1 staining. J Pathol 2005: 207 (4): 385–395.
5. Mangold E, Pagenstecher C, Friedl W et al. Spectrum and frequencies of mutations in MSH2 and MLH1 identified in 1,721 German families suspected of hereditary nonpolyposis colorectal cancer. Int J Cancer 2005: 116 (5): 692–702.
6. Barnetson RA, Tenesa A, Farrington SM et al. Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer. N Engl J Med 2006: 354 (26): 2751–2763.
7. Naseem H, Boylan J, Speake D et al. Inherited association of breast and colorectal cancer: limited role of CHEK2 compared with high-penetrance genes. Clin Genet 2006: 70 (5): 388–395.
8. Vaccaro CA, Bonadeo F, Roverano AV et al. Hereditary nonpolyposis colorectal cancer (Lynch Syndrome) in Argentina: report from a referral hospital register. Dis Colon Rectum 2007: 50 (10): 1604–1611.
9. Pinheiro M, Ahlquist T, Danielsen SA et al. Colorectal carcinomas with microsatellite instability display a different pattern of target gene mutations according to large bowel site of origin. BMC Cancer 2010: 10: 587.
10. Bonadona V, Bonaïti B, Olschwang S et al. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. JAMA 2011: 305 (22): 2304–2310.
11. Walsh MD, Buchanan DD, Pearson SA et al. Immunohistochemical testing of conventional adenomas for loss of expression of mismatch repair proteins in Lynch syndrome mutation carriers: a case series from the Australasian site of the colon cancer family registry. Mod Pathol 2012: 25 (5): 722–730.

12. Isidro G, Veiga I, Matos P et al. Four novel MSH2/MLH1 gene mutations in Portuguese HNPCC families. Hum Mutat 2000: 15 (1): 116.
13. Fidalgo P, Almeida MR, West S et al. Detection of mutations in mismatch repair genes in Portuguese families with hereditary non-polyposis colorectal cancer (HNPCC) by a multi-method approach. Eur J Hum Genet 2000: 8 (1): 49–53.
14. Cravo M, Afonso AJ, Lage P et al. Pathogenicity of missense and splice site mutations in hMSH2 and hMLH1 mismatch repair genes: implications for genetic testing. Gut 2002: 50 (3): 405–412.
15. Isidro G, Matos S, Gonçalves V et al. Novel MLH1 mutations and a novel MSH2 polymorphism identified by SSCP and DHPLC in Portuguese HNPCC families. Hum Mutat 2003: 22 (5): 419–420.
16. Lage PA, Albuquerque C, Sousa RG et al. Association of colonic and endometrial carcinomas in Portuguese families with hereditary nonpolyposis colorectal carcinoma significantly increases the probability of detecting a pathogenic mutation in mismatch repair genes, primarily the MSH2 gene. Cancer 2004: 101 (1): 172–177.
17. Sousa R, Lage P, Ferreira S et al. Need of new clinical criteria for the identification of genetic Lynch syndrome. Acta Med Port 2007: 20 (6): 535–542.
18. Nachman MW, Crowell SL. Estimate of the mutation rate per nucleotide in humans. Genetics 2000: 156 (1): 297–304.
19. Zhivotovsky LA. Estimating divergence time with the use of microsatellite genetic distances: impacts of population growth and gene flow. Mol Biol Evol 2001: 18 (5): 700–709.
20. van der Luijt RB, van Zon PH, Jansen RP, van der Sijs-Bos CJ, Wárlám-Rodenhuis CC, Ausems MG. *De novo* recurrent germline mutation of the BRCA2 gene in a patient with early onset breast cancer. J Med Genet 2001: 38 (2): 102–105.

PAPER III

Manuela Pinheiro, Carla Pinto, Ana Peixoto, Isabel Veiga, Paula Lopes, Rui Henrique, Fátima Carneiro, Raquel Seruca, Ian Tomlinson, Karl Heinimann, Manuel R.Teixeira.

"Target gene mutational pattern in Lynch syndrome colorectal carcinomas according to tumor location and germline mutation"

Manuscript in submission

Target gene mutational pattern in Lynch syndrome colorectal carcinomas according to tumor location and germline mutation

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PAPER III

ABSTRACT

We have previously reported in sporadic MSI CRC that the target genes in MMR deficient tumors in distal colon and rectum differ from those occurring elsewhere in the colon. In this study we aimed to compare the target gene mutational pattern in MSI CRC from Lynch syndrome patients stratified by tumor location, germline mutated gene and type of mutation. A series of CRC from patients with Lynch syndrome was analyzed for microsatellite instability in genes predicted to be selective target genes of MSI and known to be involved in several pathways of colorectal carcinogenesis, especially the TGFβ superfamily and WNT pathways. The most frequently mutated genes belonged to the TGFβ superfamily signaling pathways, namely the *ACVR2A* and *TGFBR2* genes. A significantly higher frequency of target gene mutations was observed in CRC from patients with germline mutations in *MLH1* or *MSH2* when compared to patients with germline *MSH6* mutations. Furthermore, CRC with somatic mutations in *MSH3* and/or *MSH6* presented a higher mean frequency of mutations in the other genes with microsatellite sequences when compared to CRC without somatic mutations in these two MMR genes. Finally, mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* genes were significantly more frequent in distal CRC in Lynch syndrome patients. Our results indicate that the pattern of genetic changes differs in colorectal carcinomas depending on the large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different pathways even if driven by generalized MSI.

INTRODUCTION

Lynch syndrome is a highly penetrant, autosomal dominant disease characterized by early-onset colorectal cancer (CRC) and extracolonic tumors of the endometrium, stomach, small bowel, ureter, renal pelvis, ovary, and hepatobiliary tract (Lynch et al, 2003). This is the most common hereditary CRC syndrome accounting for up to 4% of all CRC cases (Aaltonen et al, 1998; Hampel et al, 2008) and is commonly caused by a genetic defect affecting one of the four mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* (Lagerstedt et al, 2007). The selection of families for genetic testing is mainly based on the personal and family cancer history using the Amsterdam criteria or the Bethesda guidelines (Vasen et al, 1999; Umar et al, 2004).

Lynch syndrome-associated CRCs are usually associated with a variety of histologic features, including tumor-infiltrating lymphocytes, Crohn disease-like lymphocytic reaction, mucinous or signet ring differentiation and a medullary growth pattern. Furthermore, they occur predominantly in the proximal colon (up to 70%), even though a significant incidence of distal CRC is also described (Percesepe et al, 1997; Peel et al, 2000; Smyrk et al, 2001). Multiple differences between cancer of the proximal and distal colon with regard to clinical behavior, epidemiological, pathological and molecular features suggest that the mechanisms of colorectal carcinogenesis may differ according to tumor location (Kapiteijn et al, 2001; Fernebro et al, 2002; Christie et al, 2013). A possible explanation for this could be the different embryological origin of the large bowel, as the ascending and two thirds of transverse colon originates from the midgut and the last third of transverse, descending colon and rectum from the hindgut (Junqueira and Zago, 1997).

More than 95% of the tumors arising in carriers of MMR gene mutations present microsatellite instability (MSI) (Aaltonen et al, 1994; Dietmaier et al, 1997). MSI is characterized by a widespread instability in coding and noncoding microsatellite sequences, due to MMR deficiency (Perucho, 1996). Through the MSI pathway, colorectal cancer progression is accelerated by a rapid mutation accumulation in coding repetitive sequences of target genes with growth-related functions. In Lynch syndrome MSI CRC, mutations have been found in a number of

genes with key cellular roles, such as growth factor receptors (*TGFBR2* and *IGF2R)*, genes involved in apoptosis (*BAX*), as well as genes relevant for DNA repair (*MSH3* and *MSH6*) (Perucho, 1996; Fujiwara et al, 1998; Yagi et al, 1998; Calin et al, 2000), together with many other microsatellite mutations that are not mechanistically responsible for the behavior of tumor cells.

We have previously reported in sporadic MSI CRC that the target genes in MMR deficient tumors in distal colon and rectum differ from those occurring elsewhere in the colon (Pinheiro et al, 2010). In this study, we aimed to compare the target genes mutational pattern in MSI CRC from Lynch syndrome patients stratified by tumor location, germline mutated gene and type of mutation (founder mutations compared to other mutations in the same gene).

MATERIAL AND METHODS

Patients, samples, and DNA extraction

This study includes altogether 129 CRC from 114 patients belonging to 98 Lynch syndrome families with deleterious MMR germline mutations (Table 1). The test series includes 78 CRC (two rectal tumors were excluded from our initial series because the patients had received neoadjuvant treatment), obtained by surgical resection, from 65 patients belonging to 50 Portuguese Lynch syndrome families presenting a germline MMR gene mutation. These mutations were identified by routine genetic diagnosis during the period of 1997 to 2011 at the Genetics Department of the Portuguese Oncology Institute, Porto, Portugal, after genetic counseling and informed consent. Thirty-nine families were followed at the Portuguese Oncology Institute and 11 at the S. João Hospital, Porto, Portugal. Twenty-eight of the families fulfilled the Amsterdam criteria, whereas the remaining presented the Bethesda criteria for genetic testing. Twenty-seven (55%) families presented a pathogenic germline mutation in *MSH2*, 16 (31%) in *MLH1*, six (12%) in *MSH6* and one (2%) in *PMS2* (data not shown) (Table 1). Furthermore, eight (16%) and ten (20%) of these families presented the *MLH1* c.1896+280_oLRRFIP2:c.1750-678del or the *MSH2* c.388_389del Portuguese founder mutations, respectively (Pinheiro et al, 2011, 2013). Forty (51%) tumors belonged to patients presenting a

germline mutation in *MSH2*, 31 (40%) in *MLH1*, six (8%) in *MSH6* and one (1%) in *PMS2*. The 65 patients included 33 females and 32 males with a mean CRC diagnosis age of 46 years (range, 15-75). Clinical data was drawn from hospital records and tumor staging was performed using the American Joint Committee on Cancer (AJCC) criteria. Summary clinic-pathological data is presented in Table 1. All the large bowel regions up to the splenic flexure were considered proximal colon and after that as distal colon. All tumor samples were paraffin embedded and were reviewed by a pathologist. Peripheral blood was collected from the same patients. DNA was isolated from paraffin-embedded tumor as described by Lungu et al (1992) and from peripheral blood using standard procedures. This study was approved by the Institutional Review Board.

We analyzed an additional series that included 51 CRC samples from 49 patients belonging to 48 Lynch syndrome families from Basel, Switzerland. Twenty-six (54%) families presented germline mutations in *MLH1*, 21 (4%) in *MSH2* and 1 (2%) in *MSH6* (Table 1). The 49 patients included 27 females and 22 males and summary clinic-pathologic data is shown in Table 1. Twenty-eight (55%) tumors belonged to patients presenting a germline mutation in *MLH1*, 22 (43%) in *MSH2*, and one (2%) in *MSH6*. Thirty-three of these families fulfilled the Amsterdam criteria, whereas the remaining presented the Bethesda criteria for genetic testing (Table 1).

MMR immunohistochemical analysis

For the assessment of MLH1, MSH2, MSH6 and PMS2 immunoexpression, four µm sections were cut and placed in silanyzed slides. Immunostaining was performed using an avidin-biotin complex peroxidase method (Elite PK-6200, Vector, Burlingame, CA, USA). Briefly, after dewaxing the sections, endogenous peroxidase activity was inhibited with freshly prepared 0.5% hydrogen peroxide in distilled water for 20 min. Antigen retrieval was performed with EDTA buffer, pH8, for 40 minutes. Incubation with primary antibodies for MLH1 (Clone G168-15, BD Pharmingem, San Jose, CA, USA), MSH2 (Clone G219-1129, BD Pharmingem), MSH6 (Clone 44, BD Pharmingem) and PMS2 (Clone A16-4, Zytomed Systems, Berlin, Germany) was performed overnight at 4ºC, at dilutions 1:100, 1:300, 1:1000, and 1:50, respectively, in 1% BSA in phosphate buffer saline (PBS). All incubations were performed in a

humified chamber. Sections were developed with a peroxidase substrate solution (0.05% 3,3-diaminobenzidine tetrahydrocloride, 0.01% H_2O_2 in PBS), counterstained with hematoxylin, dehydrated and mounted. Appropriate positive and negative controls were used for each antibody. Assessment of MLH1, MSH2, MSH6 and PMS2 immunoexpression was performed by light microscopy at x400 magnification.

Microsatellite instability analysis

Microsatellite instability evaluation was performed using the Bethesda panel of markers (BAT25, BAT26, D2S123, D5S346 and D17S250) and the 1997 National Cancer Institute guidelines. PCR was carried out as previously described using fluorescence-labeled primers (Dietmaier et al, 1997). Fragments were analyzed for length variations on an ABI Prism 310 DNA sequencer (Applied Biosystems, Foster City, USA) and allele sizes were determined using the Genemapper software (version 3.7, Applied Biosystems). The results were independently scored by two observers and an additional round of analyses confirmed the results.

Target gene analyses

For somatic mutation analysis, we selected 17 genes belonging to pathways involved in CRC carcinogenesis and most of them known to present mutations in coding microsatellite sequences in tumors with MSI: *TGFBR2* (A)10, *ACVR2A* (A)8, *BMPR1A* (T)6 (two sequences), *BMPR2* (A7) and (A)11, *EGFR* (A)13, *E2F4* (CAG)13, *BAX* (G)8, *PRDM2* (A)8 and (A)9, *TCF7L2* (A)9, *APC* (A)6 and (A4), *AXIN1* (C)6 (two sequences) and (G)6, *AXIN2* (A)6, (G7), (C)5 (two sequences) and (C)6, *PTEN* (A)6 (two sequences), *MSH6* (C)8, *MSH3* (A)8, *IGF2R* (G)8, and *B2M* (CT)4 (Supplementary Table 1). *BMPR1A*, *BMPR2, PTEN*, *PRDM2*, *APC*, *AXIN1* and *AXIN2* each include two or more repeat sequences. Selected microsatellite sequences of the potential target genes were analyzed by PCR and fragment analysis, except *AXIN2*. PCR was carried out as previously described using the fluorescence-labeled primers presented in Supplementary Table 1 (Dietmaier et al, 1997; Oliveira et al, 1998). Fragments were analyzed for length variations on an ABI Prism 310 DNA sequencer and allele sizes were determined using Genemapper

software. The results were independently scored by two observers and an additional round of analyses confirmed the results. Additionally, all cases that presented length variations between tumor and matching blood samples were confirmed by direct sequencing on an ABI 310 DNA sequencer using Big Dye Terminator V1.1 Chemistry (Applied Biosystems), according to the manufacturer's recommendations. *AXIN2* microsatellite sequences were analyzed by direct sequencing.

Statistical analysis

Statistical analysis was carried out with SPSS version 22. Results were expressed in absolute frequencies and percentages. The statistical significance of association between different variables was performed using the Fisher Exact Probability Test. Analysis of variance (ANOVA) was performed to compare group means and the statistical significance among the samples was assessed using the Scheffe Multiple comparison test. *P* values inferior to 0.05 were considered statistically significant.

RESULTS

MMR immunohistochemical analyses

All tumors from the Porto test series analyzed by immunohistochemistry from patients presenting a mutation in *MLH1* gene showed absence of MLH1 and PMS2 protein expression; patients with a *MSH2* mutation showed absence of MSH2 and MSH6 protein expression; patients with a mutation in *MSH6* gene showed absence of MSH6 protein expression and one also presented loss of MSH2 protein expression; and the tumor of the patient with the *PMS2* mutation showed loss of PMS2 protein expression. In the additional series from Basel, all the CRC analyzed by immunohistochemistry from patients presenting a mutation in *MLH1* gene showed absence of MLH1, patients with a *MSH2* mutation showed absence of MSH2 protein expression and patients with a mutation in *MSH6* gene showed absence of MSH6 protein.

Overall microsatellite instability

The frequency of MSI in the test series from Porto was 99% (77/78) and the only CRC that did not present MSI belonged to a patient carrying a germline mutation in the *MSH6* gene. In the additional series from Basel all 51 Lynch syndrome CRC presented MSI.

Target gene mutation frequencies

The microsatellite sequence most frequently mutated in the test series from Porto was *ACVR2A* (90.9%), followed by *TGFBR2* (89.6%), *EGFR* (88.3%) and *BMPR2* (A)11 sequence (74%). Mutations in the *E2F4* (51.9%), *MSH3* (49.4%), *BAX* (44.2%), *TCF4* (41.6%), and *BMPR2* (A)7 microsatellite sequence (35.1%) were also frequent. The remaining genes presented a mutational frequency inferior to 30% (Table 2). All the MSI CRC presented microsatellite instability in at least one of the genes analyzed and the overall mean of mutation frequency was 6.7±2.4.

In the additional series from Basel we only analyzed the target gene sequences showing significant differences between proximal and distal CRC (see below), namely the *MSH3* (A)8 and *BMPR2* (A)7 microsatellite sequences. The mutational frequency in this series was 47.1% and 39.2% for *MSH3* and *BMPR2*, respectively, being 48.4% for *MSH3* and 36.7% for *BMPR2* in the total series.

Target gene analysis by germline MMR mutation

The mean frequency of target gene mutations was 7.1±2.1 in the tumors from patients presenting a germline mutation in *MSH2*, 6.8±2.5 in *MLH1* carriers, 3.0±1.6 in *MSH6* carriers, and the single tumor from a *PMS2* carrier had 7 mutations. In order to examine whether there were statistically significant differences among these groups a one-way ANOVA was conducted. The results revealed statistically significant differences among the groups (P=0.001). Post-hoc Scheffe tests revealed statistically significant differences between *MLH1* and *MSH6* (P=0.003), *MSH2* and *MSH6* (P= 0.001) but not between *MLH1* and *MSH2* (P=0.884) (Figure 1A).

No association was observed between the target gene mutational frequencies and pattern according to the germline mutated gene and the type of mutation, nor

when comparing founder mutations to other mutations in the same gene and CRC from the same individual.

Target gene analysis by somatic MMR mutation

CRC presenting somatic mutations in *MSH3* and/or *MSH6* harbored a higher mean frequency of mutations in the other microsatellite sequences analyzed when compared to the CRC without somatic mutations is these two MMR genes. The mean mutational frequency was 7.4±1.7 in the tumors presenting only somatic *MSH3* mutations, 7.4±2.2 when only *MSH6* was mutated, 9.4±1.2 when both *MSH3* and *MSH6* were mutated, and 5.0±2.0 when no *MSH3*/*MSH6* somatic mutation was present. The differences were statistically significant among the groups (P<0.001), and the Post-hoc Scheffe test revealed a statistically significant difference between all the groups presenting somatic mutations in *MSH3* and/or *MSH6* and the group of tumors without any of these mutations (P<0.05) (Figure 1B).

Target gene mutation analysis by tumor location

The mean frequency of target gene mutations in proximal and distal colon tumors was 6.33±2.3 and 7.42±2.4, respectively, being this difference not statistically significant. When considering tumor location, two microsatellite sequences were preferentially mutated in tumors with origin in the distal colon (Table 2). In the CRC test series from Porto, *BMPR2* (A)7 microsatellite sequence and *MSH3* mutations were present in 14 (53.8%) and 18 (69.2%) out of 26 MSI distal carcinomas, whereas in MSI proximal carcinomas mutations were present in 13 (25.5%) and 20 (39.2%) out of 51 tumors, the difference being statistically significant (P=0.022 for *BMPR2* and P=0.017 for *MSH3*) (Table 2). This result was confirmed when we enlarged the series with the group of CRC from Lynch syndrome patients from Basel (P=0.039 for *MSH3* and P=0.012 for *BMPR2*) (Figure 2). Other genes show different mutation frequencies according to the large bowel site of origin (Table 2), but their lower mutation frequencies require much large tumor series to evaluate their significance. For instance, *APC* microsatellite mutations were only detected in the proximal colon, whereas *AXIN1*, *AXIN2*, and *PTEN* microsatellite mutations only rarely were found in

the proximal CRC and were recurrently detected in distal CRC. No association was observed between target genes mutational pattern or frequency with other clinic-pathological features, namely gender, mean age of CRC diagnosis, tumor staging or differentiation grade.

DISCUSSION

CRC in Lynch syndrome patients present certain clinicohistopathologic features that differentiate them from MSI sporadic CRC, namely a younger age at diagnosis and the more frequent presence of lymphocytic infiltration and a mucinous histologic type (Young et al, 2001; Shia et al, 2003; Yearsley et al, 2006). Furthermore, although Lynch syndrome CRC with MSI tend to occur more frequently in the proximal colon, some authors have reported frequencies up to 40% of MSI distal carcinomas in Lynch syndrome patients, contrasting with sporadic MSI cancers that occur in the proximal colon in ~90% of the cases (Kim et al, 1994; Mueller-Koch et al, 2005; Moghbeli et al 2011; Moussa et al, 2011). The frequency of proximal and distal CRC in the overall series of MMR mutation carriers we here present is 67% and 33%, respectively, which confirms that a noteworthy proportion of CRC in Lynch syndrome patients occurs in distal colon. Irrespective of tumor location, it is thought that MSI is a hallmark of CRC in Lynch syndrome patients, occurring in more than 95% of the tumors (Aaltonen et al, 1994; Dietmaier et al, 1997). The frequency of microsatellite instability in the test series from Porto was 99% of the 78 CRC, with only one tumor without MSI belonging to a patient carrying a germline mutation in *MSH6*, and in the Basel additional series all 51 tumors presented MSI. Since a somewhat lower frequency of MSI has been described for *MSH6* germline carriers (Peterlongo et al, 2003; Barnetson et al, 2006), the series we here present are representative of Lynch syndrome CRC.

There is a well-established association between an ineffective MMR system and mutations in genes with key cellular roles in CRC of Lynch syndrome patients. Furthermore, it has been demonstrated that mutational inactivation of specific target genes and pathways provide a growth advantage to affected cells (Furlan et al, 2002). With the purpose of comparing the mutation pattern of target genes in MSI

CRC from Lynch syndrome patients stratified by tumor location and germline mutation type, we initially analyzed 78 tumors for microsatellite instability in genes predicted to be selective target genes of MSI and known to be involved in several pathways of colorectal carcinogenesis, especially the TGFβ superfamily and WNT pathways. All candidate genes except *BMPR1A* presented frameshift mutations in the microsatellite sequences analyzed, with a frequency that ranged from 2.6% (*AXIN1*) to 90.9% (*ACVR2A*). All MSI CRC cases presented at least one mutation in the genes analyzed and the overall mean mutation frequency was 6.7±2.4. We also observed that CRC from patients with a germline mutation in *MSH6* presented a significantly lower frequency of target gene mutations when compared to the groups with *MLH1* or *MSH2* germline mutations. Wu et al (1999) had already suggested that *MSH6* may be involved in a proportion of Lynch syndrome patients presenting MSI-low tumors. More recently, Laghi et al (2012) also observed that the mutational rate in specific target genes was significantly lower in *MSH6* than in *MLH1* and *MSH2* deficient tumors.

A considerable frequency of somatic mutations in *MSH3* and *MSH6* microsatellite sequences have been described in CRC from patients with germline mutations in MMR genes (Akiyama et al, 1997; Yamamoto et al, 1998). These mutations are considered as secondary events resulting from a germline MMR gene deficiency and are defined as "secondary" mutators in a "mutator that mutates another mutator" model (Akiyama et al, 1997; Yamamoto et al, 1998). In our study, mutations in the *MSH3* and *MSH6* mononucleotide repeats were detected in 49.4% and 24.7% of the CRC analyzed, respectively. According to the literature, their mutational frequency in Lynch syndrome CRC with MSI is 52 to 58.3% for *MSH3* and 16.7 to 34% for *MSH6* (Akiyama et al, 1997; Yamamoto et al, 1998; Miyaki et al, 2001). We observed that CRC with somatic mutations in *MSH3* and/or *MSH6* presented a higher mean frequency of mutations in the other microsatellite sequences analysed when compared to CRC without somatic mutations is these two MMR genes. These could indicate that mutations in two or more MMR genes may enhance MSI and hence the accumulation of mutations in other cancer associated genes during tumor progression (Akiyama et al, 1997; Yamamoto et al, 1998; Laghi et al, 2012). Interestingly, we observed that the *MSH3* gene was significantly more

frequently mutated in Lynch syndrome tumors with origin in distal colon. This association was confirmed when we enlarged the initial test series with the additional series from Basel, indicating that this gene is preferentially involved in the development or progression of distal colon cancer in Lynch syndrome patients.

The most frequently mutated genes belonged to the TGFβ superfamily of proteins, which includes the TGFβ, activin and BMP subfamilies. It plays a critical role in carcinogenesis via regulation of cell growth, differentiation, proliferation, and apoptosis and it is estimated that ~80% of human colorectal cancers harbor mutations affecting at least one component of TGFβ superfamily signaling (Grady et al, 1999; Blobe et al, 2000). *TGFBR2* and *ACVR2A* have been described as frequently mutated in MSI cancers, namely in Lynch MMR deficient tumors (Fujiwara et al, 1998; Miyaki et al, 2001). In our series these two genes were the most frequently mutated supporting the idea that alterations in these genes are important for the development of MSI CRC in the context of Lynch syndrome. On the other hand, the involvement of the BMP pathway in MSI CRC has scarcely been studied (Kodach et al, 2008). The BMP pathway has been implicated in the initiation of colorectal cancer among individuals with juvenile polyposis harboring *BMPR1A* mutations. BMP signaling is mediated by its receptors (BMPR1 and BMPR2) and downstream molecules such as Smad. In this study, we observed a high frequency of mutations in the microsatellite sequences of *BMPR2* (74% and 35.1% in (A)11 and (A)7, respectively). Kodach et al (2008) also found a high mutational frequency (81.4%) in the (A)11 microsatellite sequence in sporadic MMR deficient tumors and indicated that loss of BMPR2 expression is associated with microsatellite instability in this sequence. Regarding the (A)7 microsatellite sequence, they detected instability in two MSI cell lines and none in the MMR-deficient tumors, whereas none of the MSS cell lines and MMR proficient tumors analyzed presented mutations in either microsatellite sequences (Kodach et al, 2008). Furthermore, Park et al (2010) detected mutations in *BMPR2* (A)7 sequence in 13.2% of sporadic CRC associated with loss of BMPR2 expression. Further studies are needed to clarify the role of the (A)7 tract mutations in *BMPR2* function and expression, given that almost all cases presented in concomitance alterations in the more commonly mutated (A)11 tract. Kodach et al (2008) also

observed that alterations in *BMPR2* occurred in concomitance with *TGFBR2* and *ACVR2* mutations, suggesting that loss of one of these receptors is insufficient for complete pathway disruption. On the other hand, some of these microsatellite mutations may be bystander events that do not play a causal role in carcinogenesis. We also observed that the *BMPR2* (A)7 microsatellite sequence presented a statistically significant higher mutational frequency in distal Lynch syndrome CRC when compared to proximal CRC. This association was confirmed when we enlarged the test series with the additional Lynch syndrome series from Basel, suggesting that mutation at the (A)7 microsatellite sequence of *BMPR2* might confer higher selective advantage in distal CRC carcinogenesis in Lynch syndrome patients.

Some studies indicate that alterations in the WNT pathway may play an important role in the tumorigenesis of MMR deficiency tumors (Miyaki et al, 1999; Huang et al, 2004; Thorstensen et al, 2005). WNT signaling plays an important role in normal embryonic development of different tissues and regulates a variety of cellular functions including differentiation, growth, and apoptosis. We detected a low frequency of mutations in *APC*, *AXIN1*, *AXIN2* and *PTEN*, but a high frequency in *TCF7L2*. Mutations in the A9 repeat in the *TCF7L2* gene have been reported with a high frequency (33% to 58%) in Lynch syndrome CRC (Zhou et al, 2002; Yamaguchi et al, 2006). Although some studies indicate *TCF7L2* as a true target gene in colorectal carcinogenesis, others demonstrated by functional studies that mutations in the *TCF7L2* microsatellite sequence had no significant effect (Duval et al 1999; Ruckert et al, 2002). Further studies are needed to understand if alterations in this microsatellite sequence contribute to colorectal carcinogenesis or are only passenger mutations.

Recently, mutations in a polymorphic (A)13/14 repeat within the 3'UTR of *EGFR* were described in a high percentage (59 to 81%) of sporadic MSI colon cancers (Yuan et al, 2009; Deqin et al, 2013; Sarafan-Vasseur et al, 2013). Although one study demonstrated that these mutations result in increased EGFR expression through enhanced EGFR mRNA stability, the other did not observe EGFR mRNA increase (Yuan et al, 2009; Sarafan-Vasseur et al, 2013). To best of our knowledge, the presence of microsatellite instability in this sequence has not been studied in

Lynch syndrome CRC before. We observed a higher frequency of mutations (88.3%) in Lynch syndrome CRC than what has been observed in sporadic MSI CRC. Such a high mutation frequency is an indicator of selective advantage, but additional functional studies are needed to clarify if *EGFR* is a true target gene in the MSI pathway of colorectal carcinogenesis, especially in the context of Lynch syndrome.

Genes involved in cell cycle control and apoptosis were also found to be frequently mutated in Lynch syndrome CRC, namely *E2F4*, *BAX* and *PRDM2* genes. Our mutational frequency is in accordance with that detected by other studies, indicating that these are target genes of genetic instability in Lynch syndrome CRC (Sakao et al, 1998; Fujiwara et al, 1998; Yagi et al, 1998; Chadwick et al, 2000; Miyaki et al, 2001; Moriyama et al, 2002; Yamaguchi et al, 2006). Furthermore, we also detected mutations in the *IGF2R* and *B2M* genes. *IGF2R* participates in the signal transduction of TGFβ and is found to be mutated in ~24% of Lynch syndrome CRC with MSI and mutations in *B2M* gene represent an important mechanism of immune evasion in MSI colon carcinogenesis (Miyaki et al, 2001; Bicknell et al, 1996).

As mentioned above, we observed that mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* were significantly more frequent in CRC with origin in distal colon. Interestingly, the results concerning *MSH3* are different from the ones we observed in a previous study of MSI sporadic tumors, where alterations in *MSH3* were very rare in tumors of distal colon (Pinheiro et al, 2010). Furthermore, the significantly lower frequency of *TGFBR2* mutations we previously observed in sporadic MSI CRC (Pinheiro et al, 2010) was not seen in the present study with Lynch syndrome patients (Figure 3). Qualitative (type of target gene) and quantitative (number and frequency of altered target genes) differences have been observed regarding MSI target genes in different types of cancers. For instance, significant differences have been described between the MSI profiles of endometrial and colorectal cancers from Lynch syndrome patients presenting the same germline mutation. Somatic mutations in *TCF7L2* were observed in ~47% of Lynch syndrome CRC, but not in endometrial cancers (Planck et al, 2000). Similarly, *TGFBR2* mutational frequency is higher in MSI colon carcinomas (70 to 90%) than in MSI endometrial carcinoma (17 to 19%), whereas *PTEN* instability was observed more

frequently (~20%) in endometrial carcinomas as compared to MSI CRC (~5%), suggesting that biological features and functional roles of target genes may differ depending on the tissue of tumor origin (Lu et al, 1995; Myeroff et al, 1995; Fujiwara et al, 1998; Duval et al, 1999, Kuismanen et al, 2002). Our data suggest that target genes differ in colorectal carcinomas depending on large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different routes even if driven by generalized MSI (Dierssen et al, 2007). Significant differences in the spectrum of molecular alterations between MSI Lynch syndrome and sporadic CRC have been observed. *BRAF* mutations, namely the p.Val600Glu, have been identified in sporadic MSI CRC associated with *MLH1* promoter hypermethylation, but not in Lynch syndrome CCR (Wang et al, 2003; Domingo et al, 2004). Conversely, *CTNNB1* gene mutations are frequent (43%) in Lynch syndrome tumors but not in sporadic MSI CRC (Miyaki et al, 1999; Johnson et al, 2005). The differences in the molecular profiles of the two pathways are consistent with the differing carcinogenesis routes of hereditary *versus* sporadic MSI CRC, specifically the traditional adenoma-carcinoma sequence in the former and a serrated route in the latter (Jass, 2007). Furthermore, the different embryological origin or proximal and distal bowel may contribute to the molecular heterogeneity of MSI CRC, similarly to what happens in carcinogenesis of different organs in Lynch syndrome patients.

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REFERENCES

Aaltonen LA, Peltomäki P, Mecklin JP, Järvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M, Sistonen P, Hamilton SR, Kinzler KW, Vogelstein B, de la Chapelle A. 1994. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res. 54(7):1645-8.

PAPER III

Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomäki P, Chadwick RB, Kääriäinen H, Eskelinen M, Järvinen H, Mecklin JP, de la Chapelle A. 1998. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 338(21):1481-7.

Akiyama Y, Tsubouchi N, Yuasa Y. 1997. Frequent somatic mutations of hMSH3 with reference to microsatellite instability in hereditary nonpolyposis colorectal cancers. Biochem Biophys Res Commun. 236(2):248-52.

Barnetson RA, Tenesa A, Farrington SM, Nicholl ID, Cetnarskyj R, Porteous ME, Campbell H, Dunlop MG. 2006. Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer. N Engl J Med. 354(26):2751-63.

Bicknell DC, Kaklamanis L, Hampson R, Bodmer WF, Karran P. 1996. Selection for beta 2-microglobulin mutation in mismatch repair-defective colorectal carcinomas. Curr Biol. 6(12):1695-7.

Blobe GC, Schiemann WP, Lodish HF. 2000. Role of transforming growth factor beta in human disease. N Engl J Med. 342(18):1350-8.

Calin GA, Gafà R, Tibiletti MG, Herlea V, Becheanu G, Cavazzini L, Barbanti-Brodano G, Nenci I, Negrini M, Lanza G. 2000. Genetic progression in microsatellite instability high (MSI-H) colon cancers correlates with clinico-pathological parameters: A study of the TGRbetaRII, BAX, hMSH3, hMSH6, IGFIIR and BLM genes. Int J Cancer. 89(3):230-5.

Chadwick RB, Jiang GL, Bennington GA, Yuan B, Johnson CK, Stevens MW, Niemann TH, Peltomaki P, Huang S, de la Chapelle A. 2000. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. Proc Natl Acad Sci U S A. 97(6):2662-7.

Christie M, Jorissen RN, Mouradov D, Sakthianandeswaren A, Li S, Day F, Tsui C, Lipton L, Desai J, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruszkiewicz AR, Moore J, Burgess AW, Busam D, Zhao Q, Strausberg RL, Simpson AJ, Tomlinson IP, Gibbs P, Sieber OM. 2013. Different APC genotypes in proximal and distal sporadic colorectal cancers suggest distinct WNT/β-catenin signalling thresholds for tumourigenesis. Oncogene. 32(39):4675-82.

Deqin M, Chen Z, Nero C, Patel KP, Daoud EM, Cheng H, Djordjevic B, Broaddus RR, Medeiros LJ, Rashid A, Luthra R. 2012. Somatic deletions of the polyA tract in the 3' untranslated region of epidermal growth factor receptor are common in microsatellite instability-high endometrial and colorectal carcinomas. Arch Pathol Lab Med. 136(5):510-6.

Dierssen JW, de Miranda NF, Ferrone S, van Puijenbroek M, Cornelisse CJ, Fleuren GJ, van Wezel T, Morreau H. 2007. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. BMC Cancer. 22;7:33.

Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. 1997. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res. 57(21):4749-56.

Domingo E, Espín E, Armengol M, Oliveira C, Pinto M, Duval A, Brennetot C, Seruca R, Hamelin R, Yamamoto H, Schwartz S Jr. 2004. Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes Chromosomes Cancer. 39(2):138-42.

Domingo E, Niessen RC, Oliveira C, Alhopuro P, Moutinho C, Espín E, Armengol M, Sijmons RH, Kleibeuker JH, Seruca R, Aaltonen LA, Imai K, Yamamoto H, Schwartz S Jr, Hofstra RM. 2005. BRAF-V600E is not involved in the colorectal tumorigenesis

of HNPCC in patients with functional MLH1 and MSH2 genes. Oncogene. 24(24):3995-8.

Duval A, Gayet J, Zhou XP, Iacopetta B, Thomas G, Hamelin R. 1999. Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. Cancer Res. 59(17):4213-5.

Duval A, Iacopetta B, Ranzani GN, Lothe RA, Thomas G, Hamelin R. 1999. Variable mutation frequencies in coding repeats of TCF-4 and other target genes in colon, gastric and endometrial carcinoma showing microsatellite instability. Oncogene. 18(48):6806-9.

Fernández-Peralta AM, Nejda N, Oliart S, Medina V, Azcoita MM, González-Aguilera JJ. 2005. Significance of mutations in TGFBR2 and BAX in neoplastic progression and patient outcome in sporadic colorectal tumors with high-frequency microsatellite instability. Cancer Genet Cytogenet. 157(1):18-24.

Fernebro E, Halvarsson B, Baldetorp B, Nilbert M. 2002. Predominance of CIN versus MSI in the development of rectal cancer at young age. BMC Cancer. 14;2:25.

Fujiwara T, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B, Hamilton SR. 1998. Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. Am J Pathol.153(4):1063-78.

Furlan D, Casati B, Cerutti R, Facco C, Terracciano L, Capella C, Chiaravalli AM. 2002. Genetic progression in sporadic endometrial and gastrointestinal cancers with high microsatellite instability. J Pathol. 197(5):603-9.

Grady WM, Myeroff LL, Swinler SE, Rajput A, Thiagalingam S, Lutterbaugh JD, Neumann A, Brattain MG, Chang J, Kim SJ, Kinzler KW, Vogelstein B, Willson JK,

Markowitz S. 1999. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res. 59(2):320-4.

Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Clendenning M, Sotamaa K, Prior T, Westman JA, Panescu J, Fix D, Lockman J, LaJeunesse J,Comeras I, de la Chapelle A. 2008. Feasibility of screening for Lynch syndrome amongpatients with colorectal cancer. J Clin Oncol. 26(35):5783-8.

Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. 2005. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med. 352(18):1851-60.

Huang J, Zheng S, Jin SH, Zhang SZ. 2004. Somatic mutations of APC gene in carcinomas from hereditary non-polyposis colorectal cancer patients. World J Gastroenterol. 10(6):834-6.

Jass JR. 2007. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology. 50(1):113-30.

Johnson V, Lipton LR, Cummings C, Eftekhar Sadat AT, Izatt L, Hodgson SV, Talbot IC, Thomas HJ, Silver AJ, Tomlinson IP. 2005. Analysis of somatic molecular changes, clinicopathological features, family history, and germline mutations in colorectal cancer families: evidence for efficient diagnosis of HNPCC and for the existence of distinct groups of non-HNPCC families. J Med Genet. 42(10):756-62.

Junqueira LC, Zago D. 1977. Fundamentos de Embriologia Humana, 2ª edição. Editora Guanabara Koogan: 130.

PAPER III

Kapiteijn E, Liefers GJ, Los LC, Kranenbarg EK, Hermans J, Tollenaar RA, Moriya Y, van de Velde CJ, van Krieken JH. 2001. Mechanisms of oncogenesis in colon versus rectal cancer. J Pathol. 195(2):171-8.

Kodach LL, Wiercinska E, de Miranda NF, Bleuming SA, Musler AR, Peppelenbosch MP, Dekker E, van den Brink GR, van Noesel CJ, Morreau H, Hommes DW, Ten Dijke P, Offerhaus GJ, Hardwick JC. 2008. The bone morphogenetic protein pathway is inactivated in the majority of sporadic colorectal cancers. Gastroenterology. 134(5):1332-41.

Kuismanen SA, Holmberg MT, Salovaara R, de la Chapelle A, Peltomäki P. 2000. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. Am J Pathol. 156(5):1773-9.

Lagerstedt Robinson K, Liu T, Vandrovcova J, Halvarsson B, Clendenning M, Frebourg T, Papadopoulos N, Kinzler KW, Vogelstein B, Peltomäki P, Kolodner RD, Nilbert M, Lindblom A. 2007. Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. J Natl Cancer Inst. 99(4):291-9.

Laghi L, Bianchi P, Delconte G, Celesti G, Di Caro G, Pedroni M, Chiaravalli AM, Jung B, Capella C, de Leon MP, Malesci A. 2012. MSH3 protein expression and nodal status in MLH1-deficient colorectal cancers. Clin Cancer Res. 18(11):3142-53.

Lu SL, Akiyama Y, Nagasaki H, Saitoh K, Yuasa Y. 1995. Mutations of the transforming growth factor-beta type II receptor gene and genomic instability in hereditary nonpolyposis colorectal cancer. Biochem Biophys Res Commun. 216(2):452-7.

Lynch HT, de la Chapelle A. 2003. Hereditary colorectal cancer. N Engl J Med. 348(10):919-32.

Miyaki M, Iijima T, Kimura J, Yasuno M, Mori T, Hayashi Y, Koike M, Shitara N, Iwama T, Kuroki T. 1999. Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer.Cancer Res. 59(18):4506-9.

Miyaki M, Iijima T, Shiba K, Aki T, Kita Y, Yasuno M, Mori T, Kuroki T, Iwama T. 2001. Alterations of repeated sequences in 5' upstream and coding regions in colorectal tumors from patients with hereditary nonpolyposis colorectal cancer and Turcot syndrome. Oncogene. 20(37):5215-8.

Moriyama H, Sasamoto H, Kambara T, Matsubara N, Ikeda M, Baba S, Meltzer SJ, Lynch HT, Shimizu K, Tanaka N. 2002. E2F-4 mutation in hereditary non-polyposis colorectal cancer. J Exp Clin Cancer Res. 21(2):185-9.

Moussa SA, Moussa A, Kourda N, Mezlini A, Abdelli N, Zerimech F, Najjar T, Jilani SB, Porchet N, Ayed FB, Manai M, Buisine MP. 2011. Lynch syndrome in Tunisia: first description of clinical features and germline mutations. Int J Colorectal Dis. 26(4):455-67.

Mueller-Koch Y, Vogelsang H, Kopp R, Lohse P, Keller G, Aust D, Muders M, Gross M, Daum J, Schiemann U, Grabowski M, Scholz M, Kerker B, Becker I, Henke G, Holinski-Feder E. 2005. Hereditary non-polyposis colorectal cancer: clinical and molecular evidence for a new entity of hereditary colorectal cancer. Gut. 54(12):1733-40.

Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, Bang Y, Lee HY, Park JG, Lynch HT, Roberts AB, Vogelstein B, Markowitz SD. 1995. A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res. 55(23):5545-7.

PAPER III

Oliveira C, Seruca R, Seixas M, Sobrinho-Simões M. 1998. The clinicopathological features of gastric carcinomas with microsatellite instability may be mediated by mutations of different "target genes": a study of the TGFbeta RII, IGFII R, and BAX genes. Am J Pathol. 153(4):1211-9.

Park SW, Hur SY, Yoo NJ, Lee SH. 2010. Somatic frameshift mutations of bone morphogenic protein receptor 2 gene in gastric and colorectal cancers with microsatellite instability. APMIS. 118(11):824-9.

Peel DJ, Ziogas A, Fox EA, Gildea M, Laham B, Clements E, Kolodner RD, Anton-Culver H. 2000. Characterization of hereditary nonpolyposis colorectal cancer families from a population-based series of cases. J Natl Cancer Inst. 92(18):1517-22.

Percesepe A, Benatti P, Roncucci L, Sassatelli R, Fante R, Ganazzi D, Bellacosa A, Genuardi M, Neri G, Viel A, Ponz de Leon M. 1997. Survival analysis in families affected by hereditary non-polyposis colorectal cancer. Int J Cancer. 71(3):373-6.

Perucho M. 1996. Microsatellite instability: the mutator that mutates the other mutator. Nat Med. 2(6):630-1.

Peterlongo P, Nafa K, Lerman GS, Glogowski E, Shia J, Ye TZ, Markowitz AJ, Guillem JG, Kolachana P, Boyd JA, Offit K, Ellis NA. 2003. MSH6 germline mutations are rare in colorectal cancer families. Int J Cancer. 107(4):571-9.

Pinheiro M, Ahlquist T, Danielsen SA, Lind GE, Veiga I, Pinto C, Costa V, Afonso L, Sousa O, Fragoso M, Santos L, Henrique R, Lopes P, Lopes C, Lothe RA, Teixeira MR. 2010. Colorectal carcinomas with microsatellite instability display a different pattern of target gene mutations according to large bowel site of origin. BMC Cancer. 10:587.

Pinheiro M, Pinto C, Peixoto A, Veiga I, Mesquita B, Henrique R, Baptista M, Fragoso M, Sousa O, Pereira H, Marinho C, Moreira Dias L, Teixeira MR. 2011. A novel exonic rearrangement affecting MLH1 and the contiguous LRRFIP2 is a founder mutation in Portuguese Lynch syndrome families. Genet Med. 13(10):895-902.

Pinheiro M, Pinto C, Peixoto A, Veiga I, Mesquita B, Henrique R, Lopes P, Sousa O, Fragoso M, Dias LM, Baptista M, Marinho C, Mangold E, Vaccaro C, Evans DG, Farrington S, Dunlop MG, Teixeira MR. 2013. The MSH2 c.388_389del mutation shows a founder effect in Portuguese Lynch syndrome families. Clin Genet. 84(3):244-50

Planck M, Wenngren E, Borg A, Olsson H, Nilbert M. 2000. Somatic frameshift alterations in mononucleotide repeat-containing genes in different tumor types from an HNPCC family with germline MSH2 mutation. Genes Chromosomes Cancer. 29(1):33-9.

Ruckert S, Hiendlmeyer E, Brueckl WM, Oswald U, Beyser K, Dietmaier W, Haynl A, Koch C, Rüschoff J, Brabletz T, Kirchner T, Jung A. 2002. T-cell factor-4 frameshift mutations occur frequently in human microsatellite instability-high colorectal carcinomas but do not contribute to carcinogenesis. Cancer Res. 62(11):3009-13.

Sakao Y, Noro M, Sekine S, Nozue M, Hirohashi S, Itoh T, Noguchi M. 1998. Microsatellite instability and frameshift mutations in the Bax gene in hereditary nonpolyposis colorectal carcinoma. Jpn J Cancer Res. 89(10):1020-7.

Sarafan-Vasseur N, Sefrioui D, Tougeron D, Lamy A, Blanchard F, Le Pessot F, Di Fiore F, Michel P, Bézieau S, Latouche JB, Frebourg T, Sesboüé R. 2013. Genetic variations of the A13/A14 repeat located within the EGFR 3' untranslated region have no oncogenic effect in patients with colorectal cancer. BMC Cancer. 13:183.

PAPER III

Shia J, Ellis NA, Paty PB, Nash GM, Qin J, Offit K, Zhang XM, Markowitz AJ, Nafa K, Guillem JG, Wong WD, Gerald WL, Klimstra DS. 2003. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol. 27(11):1407-17.

Smyrk TC, Watson P, Kaul K, Lynch HT. 2001. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer. 91(12):2417-22.

Thorstensen L, Lind GE, Løvig T, Diep CB, Meling GI, Rognum TO, Lothe RA. 2005. Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. Neoplasia. 7(2):99-108.

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 96(4):261-8.

Vasen HF, Watson P, Mecklin JP, Lynch HT. 1999. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 116(6):1453-6.

Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, van Der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM. 1999. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. Am J Hum Genet. 65(5):1291-8.

Yagi OK, Akiyama Y, Nomizu T, Iwama T, Endo M, Yuasa Y. 1998. Proapoptotic gene BAX is frequently mutated in hereditary nonpolyposis colorectal cancers but not in adenomas. Gastroenterology. 114(2):268-74.

Yamaguchi T, Iijima T, Mori T, Takahashi K, Matsumoto H, Miyamoto H, Hishima T, Miyaki M. 2006. Accumulation profile of frameshift mutations during development and progression of colorectal cancer from patients with hereditary nonpolyposis colorectal cancer. Dis Colon Rectum. 49(3):399-406.

Yamamoto H, Sawai H, Weber TK, Rodriguez-Bigas MA, Perucho M. 1998. Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. Cancer Res. 58(5):997-1003.

Yearsley M, Hampel H, Lehman A, Nakagawa H, de la Chapelle A, Frankel WL. 2006. Histologic features distinguish microsatellite-high from microsatellite-low and microsatellite-stable colorectal carcinomas, but do not differentiate germline mutations from methylation of the MLH1 promoter. Hum Pathol. 37(7):831-8.

Young J, Simms LA, Biden KG, Wynter C, Whitehall V, Karamatic R, George J, Goldblatt J, Walpole I, Robin SA, Borten MM, Stitz R, Searle J, McKeone D, Fraser L, Purdie DR, Podger K, Price R, Buttenshaw R, Walsh MD, Barker M, Leggett BA, Jass JR. 2001. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol. 159(6):2107-16.

Yuan Z, Shin J, Wilson A, Goel S, Ling YH, Ahmed N, Dopeso H, Jhawer M, Nasser S, Montagna C, Fordyce K, Augenlicht LH, Aaltonen LA, Arango D, Weber TK, Mariadason JM. 2009. An A13 repeat within the 3'-untranslated region of epidermal growth factor receptor (EGFR) is frequently mutated in microsatellite instability colon cancers and is associated with increased EGFR expression. Cancer Res. 69(19):7811-8.

Zhou XP, Kuismanen S, Nystrom-Lahti M, Peltomaki P, Eng C. 2002. Distinct PTEN mutational spectra in hereditary non-polyposis colon cancer syndrome-related

endometrial carcinomas compared to sporadic microsatellite unstable tumors. Hum Mol Genet. 11(4):445-50.

FIGURES

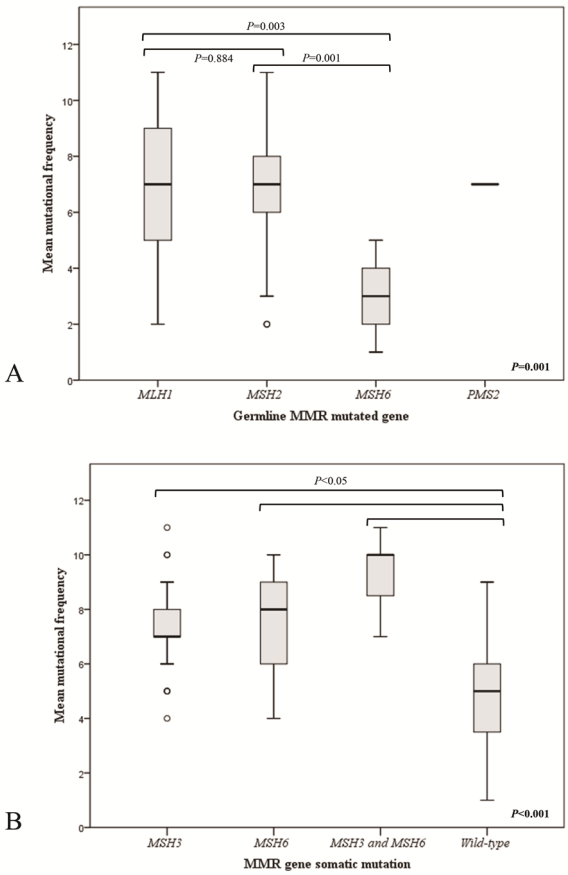

Figure 1 - (A) Box plot analyses of the mean frequency of target gene mutations (Y axis) in CRC samples from the test series categorized by MMR germline mutation (X axis). (B) Box plot analyses of the mean frequency of target gene mutations (Y axis) in CRC samples from the test series categorized by MMR somatic mutation status (X axis). The mean comparison was calculated using the One-way ANOVA test. Statistical significance among the samples was assessed using the Scheffe Multiple Comparison Test.

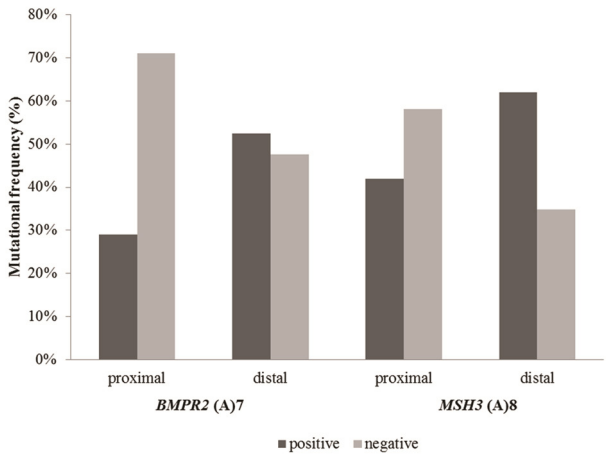

Figure 2 - Mutational frequency of the microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* genes according to tumor location in tumors from both series of Lynch syndrome patients.

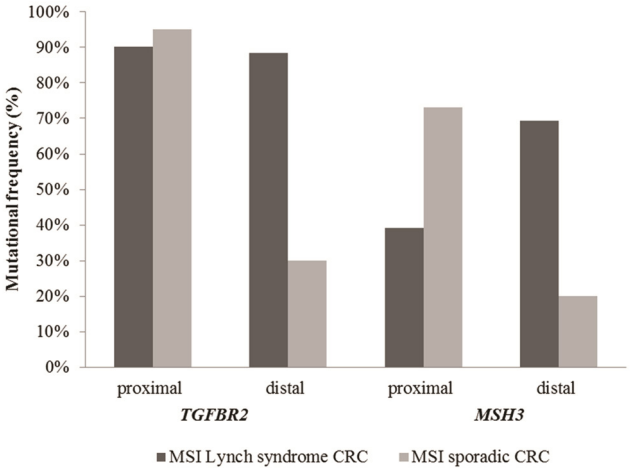

Figure 3 - Mutational frequency in *TGFBR2* (A)10 and *MSH3* (A)8 microsatellite sequences categorized by tumor location in sporadic (Pinheiro et al, 2010) and Lynch syndrome MSI CRC (present report).

TABLES

Table 1 – Clinicopathologic characteristics of the Lynch syndrome patients and families.

	Portuguese Lynch syndrome families					Basel Lynch syndrome families			
	Total	***MLH1***	***MSH2***	***MSH6***	***PMS2***	**Total**	***MLH1***	***MSH2***	***MSH6***
Families	50	16 (31%)	27 (55%)	6 (12%)	1 (2%)	48	26 (54%)	21 (44%)	1 (2%)
Criteria									
Amsterdam	28 (56%)	9 (32%)	18 (64%)	1 (4%)	0 (0%)	33 (69%)	22 (67%)	10 (30%)	1 (3%)
Bethesda	22 (44%)	7 (32%)	9 (41%)	5 (23%)	1 (5%)	15 (31%)	4 (27%)	11 (73%)	0 (0%)
Nº CRC patients	65	25 (39%)	33 (51%)	6 (9%)	1 (2%)	49	27 (55%)	21 (43 %)	1 (2%)
Gender									
Female	32 (49%)	13 (41%)	16 (50%)	2 (6%)	1 (3%)	27 (55%)	15 (56%)	11 (41%)	1 (4%)
Male	33 (51%)	12 (36%)	17 (52%)	4 (12%)	0 (0%)	22 (45%)	12 (55%)	10 (45%)	0 (0%)
CRC diagnosis age (years)									
Mean	46	46	46	44	55	na	na	na	na
Range	15-75	27-68	15-75	21-71		na	na	na	na
≤50	55 (71%)	21 (38%)	29 (53%)	5(9%)	0 (0%)	na	na	na	na
>50	23 (29%)	10 (43%)	11 (48%)	1 (4%)	1 (4%)	na	na	na	na
Total CRC	78	31 (40%)	40 (51%)	6 (8%)	1 (1%)	51	28 (55%)	22 (43%)	1 (2%)
CRC localization									
Proximal	51 (65%)	23 (45%)	24 (47%)	4 (8%)	0 (0%)	35 (69%)	22 (63%)	12 (34%)	1 (3%)
Distal	27 (35%)	8 (30%)	16 (59%)	2 (7%)	1 (4%)	16 (31%)	6 (38%)	10 (63%)	0 (0%)
TNM stage[a]									
I/II	45 (68%)	20 (44%)	23 (51%)	2 (4%)	-	32 (70%)	20 (63%)	12 (38%)	0 (0%)
III/IV	21 (32%)	9 (43%)	9 (43%)	3 (15%)	-	14 (30%)	6 (43%)	7 (50%)	1 (7%)
Differentiation grade[b]									
well/moderately	53 (90%)	20 (38%)	28 (53%)	5 (9%)	0 (0%)	25 (66%)	15 (60 %)	10 (40 %)	0 (0%)
Poorly	6 (10%)	2 (33%)	3 (50%)	0 (0%)	1 (17%)	13 (34%)	9 (69%)	4 (31%)	0 (0%)

[a] Information was not available for 12 and 5 CRC from the Portuguese and Swiss Lynch syndrome families, respectively.

[b] Information was not available for 19 and 13 CRC from the Portuguese and Swiss Lynch syndrome families, respectively.

na - not available

Table 2 – Test series mutational frequency of the target genes microsatellite sequence according to tumor location.

Gene	Total (%)	Proximal colon (%)	Distal colon (%)	*P*
ACVR2	90.9	90.2	92.3	
TGFBR2	89.6	90.2	88.5	
EGFR	88.3	88.0	92.3	
BMPR2 (A)11	74.0	72.0	80.8	
E2F4	51.9	60.0	40.0	
MSH3	49.4	39.2	69.2	0.017
BAX	44.2	41.2	50.0	
TCF7L2	41.6	37.3	50.0	
BMPR2 (A)7	35.1	25.5	53.8	0.022
RIZ	29.9	31.4	26.9	
MSH6	24.7	19.6	34.6	
IGFR2R	20.8	17.6	26.9	
B2M	9.1	11.8	3.8	
APC	7.8	11.8	0.0	
PTEN	7.8	2.0	19.2	
AXIN1	2.6	0.0	7.7	
AXIN2	3.9	2.0	7.7	

Supplementary table 1 - Target gene microsatellite sequences and primer sequences.

Target gene (mRNA Refseq)	Chromosome location	Function/Pathway	Microsatellite sequence	Exon (nucleotide position)	Primer sequence[a]
TGFBR2	3p22	Growth factor receptor	(A)10	exon 4 (c.449 to c.458)	F - AGATGCTGCCTTCCTCCAAAGTGC
(NM_001024847.2)		/TGFB			R - GTTTCTTTTGCACTCATCAGAGCTACAGG
ACVR2A	2q22.3	Growth factor receptor	(A)8	exon 4 (c.278 to c.285)	F - GTTGCCATTTGAGGAGGAAA
(NM_001278579.1)		/TGFB			R - GTTTCTTCAGCATGTTTCTGCCAATAATC
BMPR1A	10q22.3	Growth factor receptor/	(T)6	exon 4 (c.171 to c.176)	F - ATGCTTCATGGCACTGGGAT
(NM_004329.2)		TGFB/BMP			R - GTTTCTTAAGGCTTTTTGGCTTTCTGGA
			(T)6	exon 7 (c.436 to c.441)	F - GCCGAGAAAAGTCGGAGCAT
					R - GTTTCTTGAGCAAAACCAGCCATCGAA
BMPR2	2q33-q34	Growth factor receptor/	(A)11	3' UTR (c.*2891 to c.*2901)	F - ACCAGAATTAGGTCACTGAAAGA
(NM_001204.6)		TGFB/BMP			R - GTTTCTTGCTCATATGGTTGCTCTGAGGT
			(A)7	exon 12 (c.1742 to c.1748)	F - TCCATCATACTGACAGCATCG
					R - GTTTCTTTGTGGTGTTTGTGGTTGTTG
EGFR	7p12	Growth factor receptor/	(A)13[b]	3' UTR (c.*282 to c.*294)	F - GCAAGAATATTGTCCCTTTGAGCA
(NM_005228.3)		MAPK, Akt and JNK			R - GTTTCTTTTCCTTGTTGGAAGAGCCCA
E2F4	16q21-q22	transcription factor/	(CAG)13[b]	exon 7 (c.918 to c.956)	F - CAACAACACTGGACACCCGGC
(NM_001950.3)		cell cycle			R - GTTTCTTTCAAAGGAGGTAGAAGGGTTGG
BAX	19q13.3-q13.4	Proapoptotic factor/	(G)8	exon 3 (c.114 to c.121)	F - ATCCAGGATCGAGCAGGGCG
(NM_004324.3)		Apoptosis			R - GTTTCTTACTCGCTCAGCTTCTTGGTG
PRDM2 (*RIZ*)	1p36.21	transcription factor/	(A)8	exon 8 (c.4270 to c.4277)	F - GGACAGCCCAAAAGGCTTA
(NM_012231.4)		cell cycle			R - GTTTCTTTTCAAGTCGGCCTTCTGC
			(A)9	exon 8 (c.4459 to c.4467)	F - GAATAAACACGCCGCCTTCA
					R - GTTTCTTGATGAGTGTCCACCTTTCTTAGATGA
TCF7L2	10q25.3	transcription factor/	(A)9	exon 14 (c.1395 to c.1403)	F - GGGCACTGTGAAGTAAGCGA
(NM_001146274.1)		WNT signalling			R - ATCTGAAGAGGGTGGGCTGA
APC	5q21-q22	tumor suppressor	(A)6	exon 16 (c.4661 to c.4666)	F - CTGAGCCTCGATGAGCCATT
(NM_000038.5)		WNT signalling	(A)4	exon 16 (c.4679 to c.4682)	R – GTTTCTTACGTGATGACTTTGTTGGCA

[a]The GTTTCTT "pig-tail" was added to the 5′ end of all reverse primers.

[b] Polymorphic microsatellite sequence.

Supplementary table 1 (continued)

Target gene (mRNA Refseq)	Chromossome location	Function/Pathway	Microsatellite sequence	Exon (nucleotide position)	Primer sequence[a]
AXIN1	16p13.3	WNT signaling	(C)6	exon 2 (c.787 to c.792)	F - ATCTGGATACCTGCCGACCT
(NM_003502)					R - GTTTCTTCGTCGGACTCACCTGAACTC
			(C)6	exon 4 (c.1029 to c.1034)	F - CCTCCTGCTCCTCTCTGAGT
					R - GTTTCTTATTGACCTGCACGCTCTCC
			(G)6	exon 6 (c.1518 to c.1523)	F - TGGACGAGCACGTACAGC
					R - GTTTCTTTTCGCCCCTGACTTGGGTA
AXIN2	17q23q24	WNT signalling	(A)6	exon 8 (c.1920 to c.1925)	F - AACCCAGTTTCTTTCCTTCT
(NM_004655.3)			(G)7	exon 8 (c.1988 to c.1994)	R - ATCCCTGCCTCAACCTA
			(C)5	exon 8 (c.2007 to c.2011)	
			(C)6	exon 8 (c.2018 to c.2023)	
			(C)5	exon 8 (c.2128 to c.2132)	
PTEN	10q23.3	tumor suppressor/	(A)6	exon 7 (c.795 to c.800)	F - CTTTGAGTTCCCTCAGCCGT
(NM_000314.4)		WNT signalling			R - GTTTCTTTCACCAATGCCAGAGTAAGCA
			(A)6	exon 8 (c.963 to c.968)	F - GAGCGTGCAGATAATGACA
					R - GTTTCTTCCCACAAAATGTTTAATTTAAC
MSH6	2p16	Mismatch repair	(C)8	exon 5 (c.3254 to c.3261)	F - GGGGTGATGGTCCTATGTGTC
(NM_000179.2)					R - GTTTCTTTAGGCTTTGCCATTTTCCT
MSH3	5q11-q12	Mismatch repair	(A)8	exon 7 (c.1141 to c.1148)	F - ACCAGCTATCTTCTGTGCATCTC
(NM_002439.4)					R - GTTTCTTAACATTTGTTCCTCACCTG
IGF2R	6q26	Growth factor receptor	(G)8	exon 28 (c.3942 to c.3949)	F - ACAGGTCTCCTGACTCAGAA
(NM_000876.2)					R - GTTTCTTGCCGTCGGTACATGCTCAC
B2M	15q21-q22.2	MHC class I	(CT)4	exon 1 (c.37 to c.44)	F - CTGGCTTGGAGACAGGTGAC
(NM_004048.2)					R - GTTTCTTGACTCACGCTGGATAGCCTC

GENERAL DISCUSSION

The germline mutational spectrum of Lynch syndrome is highly heterogeneous considering the several types of mutations located throughout the four relevant MMR genes. However, specific mutations are observed at high frequencies in well-defined populations or ethnic groups, because of founder and or recurrent effects. The identification of founder mutations, besides increasing the cost-effectiveness of genetic testing of Lynch syndrome suspects, allows the analysis of mutation prevalence in different populations and mutation-specific effects on penetrance and disease phenotype (Chan et al, 2001; Wagner et al, 2003; Caluseriu et al, 2004; Sun et al, 2005).

Additionally, molecular characterization of CRC arising in Lynch syndrome patients, namely the genes mutated somatically and the respective disrupted pathways, is imperative to increase our understanding of the carcinogenic mechanisms, which in turn might allow the discovery of potential treatment targets and/or predictive markers.

1 - Identification of a novel exonic rearrangement affecting *MLH1* and the contiguous *LRRFIP2* that is a founder mutation in Portuguese Lynch syndrome families

Several studies have shown that large genomic rearrangements in MMR genes, mainly in *MSH2* and *MLH1*, are a frequent cause of Lynch syndrome, reaching a proportion as high as 20% (Charbonnier et al, 2002; Di Fiore et al, 2004;). Molecular characterization of large genomic rearrangements is essential to determine if it is a novel alteration and to understand the mutational mechanism responsible for its occurrence. The majority of studies with large rearrangement breakpoint identification propose that they are caused by homologous recombination between Alu elements (Moisio et al, 1996; Wang et al, 2003; Nyström et al, 1995).

GENERAL DISCUSSION

We identified a novel *MLH1* exonic rearrangement in 14 Portuguese Lynch syndrome families, accounting for ~17% of all Lynch syndrome families with pathogenic mutations identified at the Genetics Department of the Portuguese Oncology Institute of Porto at the time (data not shown). This rearrangement, detected by the Multiplex Ligation Probe Amplification (MLPA) methodology, comprised at least exons 17 to 19 of *MLH1* gene and exon 26 of the contiguous *LRRFIP2* gene. Breakpoint identification revealed that these families harbored an identical deletion involving *MLH1* exons 17 to 19 and *LRRFIP2* exons 26 to 29, corresponding to the mutation *MLH1* c.1896+279_oLRRFIP2:c.1750-678del. Analysis of the genomic sequences flanking the deletion breakpoints suggested that this large rearrangement resulted from homologous recombination between two Alu sequences present in introns 16 and 25 of the genes *MLH1* and *LRRFIP2*, respectively.

All 14 families were originated from the Porto district countryside and haplotype analysis revealed a conserved region of ~1 Mb, indicating that these families share a common ancestor. Based on the mutation and recombination events observed in microsatellite haplotypes and assuming a generation time of 25 years, the age estimate for the *MLH1* c.1896+279_oLRRFIP2:c.1750-678del mutation was 283±78 years.

Founder mutations have been identified in Lynch syndrome families from several countries and population, namely China, the United States, Italy, and among Ashkenazi Jews (Chan et al, 2001; Wagner et al, 2003; Caluseriu et al, 2004; Sun et al, 2005). A well-known founder mutation is the deletion of *MLH1* exon 16 that represents approximately 50% of all *MLH1* mutations detected in Lynch syndrome families from Finland (Moisio et al, 1996). The identification of founder mutations facilitates the molecular diagnosis of Lynch syndrome by targeting the mutational analysis to specific gene regions before full screening of all MMR genes. Screening for the *MLH1* c.1896+280_oLRRFIP2:c.1750-678del mutation as a first step may increase the cost-effectiveness of genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal.

2 - Identification of a *MSH2* founder mutation in Portuguese Lynch syndrome families but recurrent worldwide

The *MSH2* c.388_389del mutation was detected in 16 Portuguese Lynch syndrome families, explaining 16% of our Lynch syndrome families with identified germline mutations at the time (data not shown). This germline mutation had already been reported in Lynch syndrome families from Germany, Scotland, England, and Argentina (Mangold et al, 2005; Barnetson et al, 2006; Naseem and Boylan et al, 2006; Vaccaro et al, 2007). Due to the high frequency of the *MSH2* c.388_389del mutation in our series and the various reports of its presence in other countries, we aimed to evaluate if this is a founder or a recurrent mutation. All the 16 known Portuguese families were originated from the north of Portugal and the informative families shared a haplotype of ~10 Mb, indicating that the *MSH2* c.388_389del alteration is a founder mutation in Portugal with a relatively recent origin. The Lynch syndrome families with the *MSH2* c.388_389del mutation living outside Portugal that we could study, showed no clear evidence for a common ancestry nor do they reported any Portuguese ancestry. The observed haplotypes suggested that the *MSH2* c.388_389del mutation has occurred *de novo* in the background of different haplotypes worldwide, but shows a founder effect in Portugal. Haplotype analysis of various recurrent mutations demonstrated multiple origins and is in some cases associated with founder effects. For instance, the *MSH2* splice-site mutation c.942+3A>T, which was proven to be widespread in the Newfoundland population through a founder effect, has been observed in many other populations arising *de novo*, and therefore is considered a worldwide recurrent mutation with a founder effect in Newfoundland (Froggat et al, 1999; Desai et al, 2000).

Considering that this mutation appeared independently several times in the human genome, this region may be considered a hotspot within the *MSH2* gene. Mutational hotspots, such as tandemly repeated sequences, can originate recurrent mutations and different mechanisms have been proposed for the generation of gene deletions mediated by short repeats and/or palindromes in the vicinity of the mutation. In the case of the recurrent *MSH2* c.388_389del mutation we here report, a short

repeat motif (TCTCTCTC) exists immediately upstream of the deletion, so we hypothesize that a similar mechanism may be operative in its recurrent *de novo* origin (van der Luijt et al, 2001).

As indicated previously, in populations where certain mutations are frequent, especially by a founder effect, important diagnostic implications exist. The high frequency of the *MSH2* c.388_389del mutation in our Lynch syndrome families with identified germline mutations indicates that screening for this *MSH2* mutation as a first step, together with the *MLH1* founder mutation discussed above, may increase the cost-effectiveness of genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal, as the two mutation together account for a significant proportion of MMR mutations found in this region.

3 - Target gene mutational spectrum in Lynch syndrome CRC according to MMR germline mutation

The somatic molecular genetic marker associated with Lynch syndrome is MSI, occurring in more than 95% of the colorectal cancers arising in these patients due to MMR deficiency (Aaltonen et al, 1994; Dietmaier et al, 1997). Through this pathway, CRC progression is accelerated by a rapid mutation accumulation in coding repetitive sequences of target genes with growth-related functions (Perucho, 1996; Boland et al, 1998; Yamamoto et al, 1998; Yagi et al, 1998; Fujiwara et al, 1998; Calin et al, 2000).

We analyzed 78 Lynch syndrome CRC for MSI and mutations in genes predicted to be selective target genes of MSI and known to be involved in important pathways for colorectal carcinogenesis, especially the TGFβ superfamily and WNT pathways. Only one CRC did not present MSI (in the markers of the Bethesda panel and in any of the genes analyzed) and belonged to a patient with a germline mutation in the *MSH6* gene. Additionally, we observed that CRC from patients with germline mutations in *MSH6* presented a significantly lower frequency of target gene mutations when compared to the group with *MLH1* or *MSH2* germline mutations. According to

some authors, the microsatellite mutational rate in *MSH6* deficient tumors is lower than in *MLH1* and *MSH2* genes (Wu et al, 1999; Laghi et al, 2012).

A considerable frequency of somatic mutations was detected in the *MSH3* and *MSH6* microsatellite sequences. We also observed that CRC with somatic mutations in *MSH3* and/or *MSH6* presented a higher mean frequency of mutations in the other microsatellite sequences analyzed when compared to CRC without somatic mutations in these two MMR genes. Somatic mutations in microsatellite sequences in MMR genes are considered secondary events, since they result from a germline MMR gene deficiency and these somatically mutated genes are defined as "secondary" mutators in a "mutator that mutates another mutator" model. The additional inactivation of these genes may enhance MSI and hence the accumulation of mutations in other cancer-associated genes during tumor progression (Akiyama et al, 1997; Yamamoto et al, 1998; Laghi et al, 2012).

The most frequently mutated genes belonged to the TGFβ superfamily of proteins, namely *TGFBR2* and *ACVR2A*, supporting the idea that alterations in these genes are important for the development of MSI CRC in the context of Lynch syndrome (Fujiwara et al, 1998; Miyaki et al, 2001). We also observed a high frequency of mutations in the microsatellite sequences of *BMPR2* gene that belongs to the BMP pathway. Regarding the WNT signaling pathway, we detected a low frequency of mutations in all genes except in *TCF7L2*. A high frequency of mutations was also detected in genes involved in cell cycle control and apoptosis, namely the *E2F4*, *BAX* and *PRDM2* genes. To our knowledge, this is the first study that analyzed the presence of microsatellite instability in the (A)13/14 sequence of *EGFR* gene in Lynch syndrome CRC and a very high frequency of mutations was found.

However, besides the differences related to the mean number of somatically mutated genes mentioned above, no significant association was seen regarding the mutational frequency and pattern of particular target genes with the germline mutated gene or the type of mutation, namely when comparing founder mutations to other mutations in the same gene.

4 - Target gene mutational spectrum in Lynch syndrome CRC according to large bowel location

Multiple differences between cancer of the proximal and distal colon with regard to clinical behavior, epidemiological, pathological and molecular features suggest that the mechanisms of colorectal carcinogenesis may differ according to tumor location (Kapiteijn et al, 2001; Fernebro et al, 2002; Christie et al, 2012).

We observed that mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* were significantly more frequent in CRC with origin in distal colon. This association was confirmed when we enlarged the initial test series with the additional series from Basel, indicating that these two genes are preferentially involved in the development or progression of distal colon cancer in Lynch syndrome patients.

Interestingly, the results concerning *MSH3* are different from the ones we observed in a previous study of MSI sporadic tumors, where alterations in *MSH3* were very rare in tumors of distal colon (Pinheiro et al, 2010). Furthermore, the significantly lower frequency of *TGFBR2* mutations we previously observed in sporadic MSI CRC (Pinheiro et al, 2010) was not seen in the present study with Lynch syndrome patients. Qualitative (type of target gene) and quantitative (number and frequency of altered target genes) differences have been observed regarding MSI target genes in different types of cancers. For instance, significant differences have been described between the MSI profiles of endometrial and colorectal cancers from Lynch syndrome patients presenting the same germline mutation regarding somatic mutations in *TCF7L2*, *TGFBR2* and *PTEN*, suggesting that biological features and functional roles of target genes may differ depending on the tissue of tumor origin (Fujiwara et al, 1998; Duval et al, 1999; Planck et al, 2000; Zhou et al, 2002; Kuismanen et al, 2002). Our data suggests that target genes differ in CRC depending on large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different routes even if driven by generalized MSI (Dierssen et al, 2007). Significant differences in the spectrum of molecular alterations between MSI Lynch syndrome and sporadic CRC have been observed. *BRAF* mutations, namely the p.Val600Glu, have been identified in sporadic

MSI CRC associated with *MLH1* promoter hypermethylation, but not in Lynch syndrome CCR (Wang et al, 2003; Domingo et al, 2004). Conversely, *CTNNB1* gene mutations are frequent (43%) in Lynch syndrome tumors but not in sporadic MSI CRC (Miyaki et al, 1999; Johnson et al, 2005). The differences in the molecular profiles of the two pathways are consistent with the differing carcinogenesis routes of hereditary *versus* sporadic MSI CRC, specifically the traditional adenoma-carcinoma sequence in the former and a serrated route in the latter (Jass, 2007). Furthermore, the different embryological origin or proximal and distal bowel may contribute to the molecular heterogeneity of MSI CRC, similarly to what happens in carcinogenesis of different organs in Lynch syndrome patients.

CONCLUSIONS

The main conclusions of this thesis are:

1. The novel exonic rearrangement *MLH1* c.1896+279_oLRRFIP2:c.1750-678del and the point mutation *MSH2* c.388_389del mutation are two frequent Portuguese frequent founder mutations, the latter being a recurrent mutation worldwide.
2. Screening for these two mutations as a first step may increase the cost-effectiveness of genetic testing of Lynch syndrome suspects of Portuguese ancestry, especially those originating from the north of Portugal.
3. Genes belonging to the TGFβ superfamily signaling pathways play an important role in the carcinogenesis of Lynch syndrome CRC, as they are the most frequently mutated.
4. CRC from patients with germline mutations in *MLH1* or *MSH2* present a significantly higher frequency of target gene mutations when compared to patients with germline *MSH6* mutations.
5. CRC with somatic mutations in *MSH3* and/or *MSH6* present a higher mean frequency of mutations in the other genes with microsatellite sequences when compared to CRC without somatic mutations in these two MMR genes.
6. Mutations in microsatellite sequences (A)7 of *BMPR2* and (A)8 of *MSH3* are significantly more frequent in CRC with origin in distal colon in Lynch syndrome patients.
7. The pattern of genetic changes differs in colorectal carcinomas depending on the large bowel site of origin and between Lynch syndrome and sporadic MSI CRC, suggesting that carcinogenesis can occur by different pathways even if driven by generalized MSI.

FUTURE PERSPECTIVES

Although MSI CRC are thought to be characterized by stable, near-diploid karyotypes, it is not known to what extent copy number neutral chromosomal changes, like those associated with uniparental disomy, are involved in colorectal carcinogenesis in Lynch syndrome patients and whether or not there are differences according to germline mutated gene or between proximal and distal colon. To address this issue, we will use the OncoScan™ FFPE Assay (Affymetrix, Santa Clara, USA) to simultaneously detect genome-wide copy number changes and allelic imbalances, as well as somatic point mutations in nine genes (*BRAF*, *KRAS*, *EGFR*, *IDH1*, *IDH2*, *PTEN*, *PIK3CA*, *NRAS*, *TP53*), in 46 CRC divided equally by proximal and distal colon location and belonging to patients with different types of MMR germline mutations.

REFERENCES

Aaltonen LA, Peltomäki P, Mecklin JP, Järvinen H, Jass JR, Green JS, Lynch HT, Watson P, Tallqvist G, Juhola M, Sistonen P, Hamilton SR, Kinzler KW, Vogelstein B, de la Chapelle A. 1994. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res. 54(7):1645-8.

Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomäki P, Chadwick RB, Kääriäinen H, Eskelinen M, Järvinen H, Mecklin JP, de la Chapelle A. 1998. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 338(21):1481-7.

Aissi S, Buisine MP, Zerimech F, Kourda N, Moussa A, Manai M, Porchet N. 2014. TP53 mutations in colorectal cancer from Tunisia: relationships with site of tumor origin, microsatellite instability and KRAS mutations. Mol Biol Rep. 41(3):1807-13.

Akiyama Y, Iwanaga R, Saitoh K, Shiba K, Ushio K, Ikeda E, Iwama T, Nomizu T, Yuasa Y. 1995. Transforming growth factor beta type II receptor gene mutations in adenomas from hereditary nonpolyposis colorectal cancer. Gastroenterology. 112(1):33-9.

Akiyama Y, Tsubouchi N, Yuasa Y. 1997. Frequent somatic mutations of hMSH3 with reference to microsatellite instability in hereditary nonpolyposis colorectal cancers. Biochem Biophys Res Commun. 236(2):248-52.

Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, Hodges AK, Davies DR, David SS, Sampson JR, Cheadle JP. 2002. Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors. Nat Genet. 30(2):227-32.

REFERENCES

Arnold CN, Goel A, Compton C, Marcus V, Niedzwiecki D, Dowell JM, Wasserman L, Inoue T, Mayer RJ, Bertagnolli MM, Boland CR. 2004. Evaluation of microsatellite instability, hMLH1 expression and hMLH1 promoter hypermethylation in defining the MSI phenotype of colorectal cancer. Cancer Biol Ther. 3(1):73-8.

Arzimanoglou II, Gilbert F, Barber HR. 1998. Microsatellite instability in human solid tumors. Cancer. 82(10):1808-20.

Astin M, Griffin T, Neal RD, Rose P, Hamilton W. 2011. The diagnostic value of symptoms for colorectal cancer in primary care: a systematic review. Br J Gen Pract. 61(586):e231-43.

Barnetson RA, Tenesa A, Farrington SM, Nicholl ID, Cetnarskyj R, Porteous ME, Campbell H, Dunlop MG. 2006. Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer. N Engl J Med. 354(26):2751-63.

Beaugerie L, Svrcek M, Seksik P, Bouvier AM, Simon T, Allez M, Brixi H, Gornet JM, Altwegg R, Beau P, Duclos B, Bourreille A, Faivre J, Peyrin-Biroulet L, Fléjou JF, Carrat F; CESAME Study Group. 2013. Risk of colorectal high-grade dysplasia and cancer in a prospective observational cohort of patients with inflammatory bowel disease. Gastroenterology. 145(1):166-175.

Berg M, Danielsen SA, Ahlquist T, Merok MA, Ågesen TH, Vatn MH, Mala T, Sjo OH, Bakka A, Moberg I, Fetveit T, Mathisen Ø, Husby A, Sandvik O, Nesbakken A, Thiis-Evensen E, Lothe RA. 2010. DNA sequence profiles of the colorectal cancer critical gene set KRAS-BRAF-PIK3CA-PTEN-TP53 related to age at disease onset. PLoS One. 5(11):e13978.

Bocker T, Schlegel J, Kullmann F, Stumm G, Zirngibl H, Epplen JT, Rüschoff J. 1996. Genomic instability in colorectal carcinomas: comparison of different evaluation methods and their biological significance. J Pathol. 179(1):15-9.

Bogaert J, Prenen H. 2014. Molecular genetics of colorectal cancer. Ann Gastroenterol. 2014;27(1):9-14.

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S. 1998. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 58(22):5248-57.

Bongaerts BW, van den Brandt PA, Goldbohm RA, de Goeij AF, Weijenberg MP. 2008. Alcohol consumption, type of alcoholic beverage and risk of colorectal cancer at specific subsites. Int J Cancer. 123(10):2411-7.

Bosman, FT, Carneiro F, Hruban RH, Theise ND. 2010. WHO Classification of Tumours of the Digestive System. WHO Classification of Tumours, Volume 3, 4th edition. IARC.

Bresalier R. 2010. Colorectal cancer. In Sleisenger MH, Feldman M, Friedman LS, Brandt LJ. Sleisenger and Fordtran's Gastrointestinal and Liver Disease, 9th edition. Saunders/Elsevier. pp. 2191-2238.

Burgess AW. Regulation of the Ras signalling network. Bioessays.1994 Jul;16(7):489-96.

Caldés T, Godino J, Sanchez A, Corbacho C, De la Hoya M, Lopez Asenjo J, Saez C, Sanz J, Benito M, Ramon Y Cajal S, Diaz-Rubio E. 2004. Immunohistochemistry and microsatellite instability testing for selecting MLH1, MSH2 and MSH6 mutation carriers in hereditary non-polyposis colorectal cancer. Oncol Rep. 12(3):621-9.

Calin GA, Gafà R, Tibiletti MG, Herlea V, Becheanu G, Cavazzini L, Barbanti-Brodano G, Nenci I, Negrini M, Lanza G. 2000. Genetic progression in microsatellite

instability high (MSI-H) colon cancers correlates with clinico-pathological parameters: A study of the TGRbetaRII, BAX, hMSH3, hMSH6, IGFIIR and BLM genes. Int J Cancer. 89(3):230-5.

Calistri D, Rengucci C, Seymour I, Lattuneddu A, Polifemo AM, Monti F, Saragoni L, Amadori D. 2005. Mutation analysis of p53, K-ras, and BRAF genes in colorectal cancer progression. J Cell Physiol. 204(2):484-8.

Caluseriu O, Di Gregorio C, Lucci-Cordisco E, Santarosa M, Trojan J, Brieger A, Benatti P, Pedroni M, Colibazzi T, Bellacosa A, Neri G, Ponz de Leon M, Viel A, Genuardi M. 2004. A founder MLH1 mutation in families from the districts of Modena and Reggio-Emilia in northern Italy with hereditary non-polyposis colorectal cancer associated with protein elongation and instability. J Med Genet. 41(3):e34.

Camps J, Armengol G, del Rey J, Lozano JJ, Vauhkonen H, Prat E, Egozcue J, Sumoy L, Knuutila S, Miró R. 2006. Genome-wide differences between microsatellite stable and unstable colorectal tumors. Carcinogenesis. 27(3):419-28.

Cancer Research UK, http://www.cancerresearchuk.org/cancer-info/cancerstats/types/skin/incidence, May 2014.

Center MM, Jemal A, Ward E. 2009. International trends in colorectal cancer incidence rates. Cancer Epidemiol Biomarkers Prev. 18(6):1688-94.

Chan DS, Lau R, Aune D, Vieira R, Greenwood DC, Kampman E, Norat T. 2011. Red and processed meat and colorectal cancer incidence: meta-analysis of prospective studies. PLoS One. 6(6)

Chan TL, Yuen ST, Ho JW, Chan AS, Kwan K, Chung LP, Lam PW, Tse CW, Leung SY. 2001. A novel germline 1.8-kb deletion of hMLH1 mimicking alternative splicing: a founder mutation in the Chinese population. Oncogene. 20(23):2976-81.

Chao A, Connell CJ, Jacobs EJ, McCullough ML, Patel AV, Calle EE, Cokkinides VE, Thun MJ. 2004. Amount, type, and timing of recreational physical activity in relation to colon and rectal cancer in older adults: the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiol Biomarkers Prev. 13(12):2187-95.

Charbonnier F, Olschwang S, Wang Q, Boisson C, Martin C, Buisine MP, Puisieux A, Frebourg T. 2002. MSH2 in contrast to MLH1 and MSH6 is frequently inactivated by exonic and promoter rearrangements in hereditary nonpolyposis colorectal cancer. Cancer Res. 2002. 62(3):848-53.

Chen JM, Cooper DN, Férec C, Kehrer-Sawatzki H, Patrinos GP. 2010. Genomic rearrangements in inherited disease and cancer. Semin Cancer Biol. 20(4):222-33.

Christie M, Jorissen RN, Mouradov D, Sakthianandeswaren A, Li S, Day F, Tsui C, Lipton L, Desai J, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruszkiewicz AR, Moore J, Burgess AW, Busam D, Zhao Q, Strausberg RL, Simpson AJ, Tomlinson IP, Gibbs P, Sieber OM. 2013. Different APC genotypes in proximal and distal sporadic colorectal cancers suggest distinct WNT/β-catenin signalling thresholds for tumourigenesis. Oncogene. 32(39):4675-82.

Cirillo L, Urso ED, Parrinello G, Pucciarelli S, Moneghini D, Agostini M, Nitti D, Nascimbeni R. 2013. High risk of rectal cancer and of metachronous colorectal cancer in probands of families fulfilling the Amsterdam criteria. Ann Surg. 257(5):900-4.

Cunningham JM, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, Thibodeau SN. 1998. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. Cancer Res.1;58(15):3455-60.

REFERENCES

Curtin K, Slattery ML, Samowitz WS. 2011. CpG island methylation in colorectal cancer: past, present and future. Patholog Res Int. 2011:902674.

Czito BG, Willett CG. 2012. Colon Cancer. In Gunderson LL, Tepper JE. Clinical Radiation Oncology, 3rd edition. Saunders. pp. 975-987.

Dai Z, Xu YC, Niu L. 2007. Obesity and colorectal cancer risk: a meta-analysis of cohort studies. World J Gastroenterol. 13(31):4199-206.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. 2002. Mutations of the BRAF gene in human cancer. Nature. 417(6892):949-54.

De Benedetti L, Varesco L, Pellegata NS, Losi L, Gismondi V, Casarino L, Sciallero S, Bonelli L, Biticchi R, Bafico A. 1993. Genetic events in sporadic colorectal adenomas: K-ras and p53 heterozygous mutations are not sufficient for malignant progression. Anticancer Res. 13(3):667-70.

Derynck R, Akhurst RJ, Balmain A. 2001. TGF-beta signaling in tumor suppression and cancer progression. Nat Genet. 29(2):117-29.

Desai DC, Lockman JC, Chadwick RB, Gao X, Percesepe A, Evans DG, Miyaki M, Yuen ST, Radice P, Maher ER, Wright FA, de La Chapelle A. 2000. Recurrent germline mutation in MSH2 arises frequently de novo. J Med Genet. 37(9):646-52.

Di Fiore F, Charbonnier F, Martin C, Frerot S, Olschwang S, Wang Q, Boisson C, Buisine MP, Nilbert M, Lindblom A, Frebourg T. 2004. Screening for genomic rearrangements of the MMR genes must be included in the routine diagnosis of HNPCC. J Med Genet. 41(1):18-20.

Diep CB, Kleivi K, Ribeiro FR, Teixeira MR, Lindgjaerde OC, Lothe RA. 2006. The order of genetic events associated with colorectal cancer progression inferred from meta-analysis of copy number changes. Genes Chromosomes Cancer. 45(1):31-41.

Diergaarde B, van Geloof WL, van Muijen GN, Kok FJ, Kampman E. 2003. Dietary factors and the occurrence of truncating APC mutations in sporadic colon carcinomas: a Dutch population-based study. Carcinogenesis. 24(2):283-90.

Dierssen JW, de Miranda NF, Ferrone S, van Puijenbroek M, Cornelisse CJ, Fleuren GJ, van Wezel T, Morreau H. 2007. HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. BMC Cancer. 22;7:33.

Dietmaier W, Wallinger S, Bocker T, Kullmann F, Fishel R, Rüschoff J. 1997. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res. 57(21):4749-56.

Domingo E, Espín E, Armengol M, Oliveira C, Pinto M, Duval A, Brennetot C, Seruca R, Hamelin R, Yamamoto H, Schwartz S Jr. 2004. Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes Chromosomes Cancer. 39(2):138-42.

Domingo E, Niessen RC, Oliveira C, Alhopuro P, Moutinho C, Espín E, Armengol M, Sijmons RH, Kleibeuker JH, Seruca R, Aaltonen LA, Imai K, Yamamoto H, Schwartz S Jr, Hofstra RM. 2005. BRAF-V600E is not involved in the colorectal tumorigenesis

of HNPCC in patients with functional MLH1 and MSH2 genes. Oncogene. 24(24):3995-8.

Durko L, Malecka-Panas E. 2014. Lifestyle modifications and colorectal cancer. Curr Colorectal Cancer Rep. 10:45-54.

Duval A, Gayet J, Zhou XP, Iacopetta B, Thomas G, Hamelin R. 1999. Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability. Cancer Res. 59(17):4213-5.

Duval A, Iacopetta B, Ranzani GN, Lothe RA, Thomas G, Hamelin R. 1999. Variable mutation frequencies in coding repeats of TCF-4 and other target genes in colon, gastric and endometrial carcinoma showing microsatellite instability. Oncogene. 18(48):6806-9.

Dyson JK, Rutter MD. 2012. Colorectal cancer in inflammatory bowel disease: what is the real magnitude of the risk? World J Gastroenterol. 18(29):3839-48.

Eaden JA, Abrams KR, Mayberry JF. 2001. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut. 48(4):526-35.

Elliott RL, Blobe GC. 2005. Role of transforming growth factor Beta in human cancer. J Clin Oncol. 23(9):2078-93.

Farber RA, Petes TD, Dominska M, Hudgens SS, Liskay RM. 1994. Instability of simple sequence repeats in a mammalian cell line. Hum Mol Genet. 3(2):253-6.

Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. Cell. 61(5):759-67.

Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray, F. 2013. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 11 [Internet]. Lyon, France: International Agency for Research on Cancer. Available from: http://globocan.iarc.fr, accessed on 06/05/2014.

Fernández-Peralta AM, Nejda N, Oliart S, Medina V, Azcoita MM, González-Aguilera JJ. 2005. Significance of mutations in TGFBR2 and BAX in neoplastic progression and patient outcome in sporadic colorectal tumors with high-frequency microsatellite instability. Cancer Genet Cytogenet. 157(1):18-24.

Fernebro E, Halvarsson B, Baldetorp B, Nilbert M. 2002. Predominance of CIN versus MSI in the development of rectal cancer at young age. BMC Cancer. 14;2:25.

Forster S, Sattler HP, Hack M, Romanakis K, Rohde V, Seitz G, Wullich B. 1998. Microsatellite instability in sporadic carcinomas of the proximal colon association with diploid DNA content, negative protein expression of p53, and distinct histomorphologic features. Surgery. 123(1):13-8.

Froggatt NJ, Green J, Brassett C, Evans DG, Bishop DT, Kolodner R, Maher ER. 1999. A common MSH2 mutation in English and North American HNPCC families: origin, phenotypic expression, and sex specific differences in colorectal cancer. J Med Genet. 36(2):97-102.

Fry RD, Mahmoud NN, Maron DJ, Bleier JIS. 2012 Colon and Rectum. In: Townsend CM, Beauchamp RD, Evers BM, Mattox KL. Sabiston Textbook of Surgery: The Biological Basis of Modern Surgical Practice, 19th edition. Elsevier Saunders. pp. 1294-1380.

Fujiwara T, Stolker JM, Watanabe T, Rashid A, Longo P, Eshleman JR, Booker S, Lynch HT, Jass JR, Green JS, Kim H, Jen J, Vogelstein B, Hamilton SR. 1998.

REFERENCES

Accumulated clonal genetic alterations in familial and sporadic colorectal carcinomas with widespread instability in microsatellite sequences. Am J Pathol.153(4):1063-78.

Gammon A, Jasperson K, Kohlmann W, Burt RW. 2009. Hamartomatous polyposis syndromes. Best Pract Res Clin Gastroenterol. 23(2):219-31.

Gervaz P, Cerottini JP, Bouzourene H, Hahnloser D, Doan CL, Benhattar J, Chaubert P, Secic M, Gillet M, Carethers JM. 2002. Comparison of microsatellite instability and chromosomal instability in predicting survival of patients with T3N0 colorectal cancer. Surgery. 131(2):190-7.

Goldblatt J, Walpole I, Robin SA, Borten MM, Stitz R, Searle J, McKeone D, Fraser L, Purdie DR, Podger K, Price R, Buttenshaw R, Walsh MD, Barker M, Leggett BA, Jass JR. 2001. Features of colorectal cancers with high-level microsatellite instability occurring in familial and sporadic settings: parallel pathways of tumorigenesis. Am J Pathol. 159(6):2107-16.

Grady WM, Ulrich CM. 2007. DNA alkylation and DNA methylation: cooperating mechanisms driving the formation of colorectal adenomas and adenocarcinomas? Gut. 56(3):318-20.

Grady WM. 2007. CIMP and colon cancer gets more complicated. Gut. 56(11):1498-500.

Green DR, Kroemer G. 2009. Cytoplasmic functions of the tumour suppressor p53. Nature. 458(7242):1127-30.

Gu W, Zhang F, Lupski JR. 2008. Mechanisms for human genomic rearrangements. Pathogenetics. 1(1):4. doi: 10.1186/1755-8417-1-4.

Guerrero S, Casanova I, Farré L, Mazo A, Capellà G, Mangues R. 2009. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation or proto-oncogene overexpression. Cancer Res. 60(23):6750-6.

Haggar FA, Boushey RP. 2009. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. Clin Colon Rectal Surg. 22(4):191-7.

Half E, Bercovich D, Rozen P. 2009. Familial adenomatous polyposis. Orphanet J Rare Dis. 12;4:22.

Hamilton W, Sharp D. 2004. Diagnosis of colorectal cancer in primary care: the evidence base for guidelines. Fam Pract. 21(1):99-106.

Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Clendenning M, Sotamaa K, Prior T, Westman JA, Panescu J, Fix D, Lockman J, LaJeunesse J, Comeras I, de la Chapelle A. 2008. Feasibility of screening for Lynch syndrome amongpatients with colorectal cancer. J Clin Oncol. 26(35):5783-8.

Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, Nakagawa H, Sotamaa K, Prior TW, Westman J, Panescu J, Fix D, Lockman J, Comeras I, de la Chapelle A. 2005. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med. 352(18):1851-60.

Hardingham JE, Butler WJ, Roder D, Dobrovic A, Dymock RB, Sage RE, Roberts-Thomson IC. 1998. Somatic mutations, acetylator status, and prognosis in colorectal cancer. Gut. 42(5):669-72.

Hemminki K, Chen B. 2004. Familial risk for colorectal cancers are mainly due to heritable causes. Cancer Epidemiol Biomarkers Prev. 13(7):1253-6.

REFERENCES

Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB. 1998. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A. 95(12):6870-5.

Huang J, Zheng S, Jin SH, Zhang SZ. 2004. Somatic mutations of APC gene in carcinomas from hereditary non-polyposis colorectal cancer patients. World J Gastroenterol. 10(6):834-6.

Iacopetta BJ, Welch J, Soong R, House AK, Zhou XP, Hamelin R. 1998. Mutation of the transforming growth factor-beta type II receptor gene in right-sided colorectal cancer: relationship to clinicopathological features and genetic alterations. JPathol.184(4):390-5.

Ilyas M, Straub J, Tomlinson IP, Bodmer WF. 1999. Genetic pathways in colorectal and other cancers. Eur J Cancer. 35(14):1986-2002.

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. 1993. Ubiquitous somaticmutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature. 363(6429):558-61.

Isobe M, Emanuel BS, Givol D, Oren M, Croce CM. 1986. Localization of gene for human p53 tumour antigen to band 17p13. Nature. 320(6057):84-5.

Jass JR, Barker M, Fraser L, Walsh MD, Whitehall VL, Gabrielli B, Young J, Leggett BA. 2003. APC mutation and tumour budding in colorectal cancer. J Clin Pathol. 56(1):69-73.

Jass JR. 2007. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology. 50(1):113-30.

Johnson V, Lipton LR, Cummings C, Eftekhar Sadat AT, Izatt L, Hodgson SV, Talbot IC, Thomas HJ, Silver AJ, Tomlinson IP. 2005. Analysis of somatic molecular changes, clinicopathological features, family history, and germline mutations in colorectal cancer families: evidence for efficient diagnosis of HNPCC and for the existence of distinct groups of non-HNPCC families. J Med Genet. 42(10):756-62.

Jones AM, Douglas EJ, Halford SE, Fiegler H, Gorman PA, Roylance RR, Carter NP, Tomlinson IP. 2005. Array-CGH analysis of microsatellite-stable, near-diploid bowel cancers and comparison with other types of colorectal carcinoma. Oncogene. 24(1):118-29.

Jung B, Doctolero RT, Tajima A, Nguyen AK, Keku T, Sandler RS, Carethers JM. 2004. Loss of activin receptor type 2 protein expression in microsatellite unstable colon cancers. Gastroenterology. 126(3):654-9.

Jung B, Smith EJ, Doctolero RT, Gervaz P, Alonso JC, Miyai K, Keku T, Sandler RS, Carethers JM. 2006. Influence of target gene mutations on survival, stage and histology in sporadic microsatellite unstable colon cancers. Int J Cancer. 118(10):2509-13.

Junqueira LC, Zago D. 1977. Fundamentos de Embriologia Humana, 2ª edição. Editora Guanabara Koogan: 130.

Kambara T, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA. 2004. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. Gut. 53(8):1137-44.

Kapiteijn E, Liefers GJ, Los LC, Kranenbarg EK, Hermans J, Tollenaar RA, Moriya Y, van de Velde CJ, van Krieken JH. 2001. Mechanisms of oncogenesis in colon versus rectal cancer. J Pathol. 195(2):171-8.

REFERENCES

Kim H, Jen J, Vogelstein B, Hamilton SR. 1994. Clinical and pathological characteristics of sporadic colorectal carcinomas with DNA replication errors in microsatellite sequences. Am J Pathol. 145(1):148-56.

Kim JH, Rhee YY, Bae JM, Kwon HJ, Cho NY, Kim MJ, Kang GH. 2013. Subsets of microsatellite-unstable colorectal cancers exhibit discordance between the CpG island methylator phenotype and MLH1 methylation status. Mod Pathol. 26(7):1013-22.

Koi M, Umar A, Chauhan DP, Cherian SP, Carethers JM, Kunkel TA, Boland CR. 1995. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res. 1994 Aug15;54(16):4308-12.

Koinuma K, Shitoh K, Miyakura Y, Furukawa T, Yamashita Y, Ota J, Ohki R, Choi YL, Wada T, Konishi F, Nagai H, Mano H. 2004. Mutations of BRAF are associated with extensive hMLH1 promoter methylation in sporadic colorectal carcinomas. Int J Cancer. 108(2):237-42.

Koornstra JJ, Mourits MJ, Sijmons RH, Leliveld AM, Hollema H, Kleibeuker JH. 2009. Management of extracolonic tumours in patients with Lynch syndrome. Lancet Oncol. 10(4):400-8.

Kouvidou Ch, Latoufis C, Lianou E, Kouvatseas G, Kakouri E, Anagnostakis D, Vrettou-Aravani V, Betsi E, Karatapanis S. Expression of Smad4 and TGF-beta2 in colorectal carcinoma. Anticancer Res. 2006 Jul-Aug;26(4B):2901-7.Maruta H,

Kuismanen SA, Holmberg MT, Salovaara R, de la Chapelle A, Peltomäki P. 2000. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. Am J Pathol. 156(5):1773-9.

Kuismanen SA, Moisio AL, Schweizer P, Truninger K, Salovaara R, Arola J, Butzow R, Jiricny J, Nyström-Lahti M, Peltomäki P. 2002. Endometrial and colorectal tumors from patients with hereditary nonpolyposis colon cancer display different patterns of microsatellite instability. Am J Pathol. 160(6):1953-8.

Laghi L, Bianchi P, Delconte G, Celesti G, Di Caro G, Pedroni M, Chiaravalli AM, Jung B, Capella C, de Leon MP, Malesci A. 2012. MSH3 protein expression and nodal status in MLH1-deficient colorectal cancers. Clin Cancer Res. 18(11):3142-53.

Larsen IK, Bray F. 2010. Trends in colorectal cancer incidence in Norway 1962-2006: an interpretation of the temporal patterns by anatomic subsite. Int J Cancer. 126(3):721-32.

Levi F, Randimbison L, Blanc-Moya R, Maspoli-Conconi M, Rosato V, Bosetti C, La Vecchia C. 2013. High constant incidence of second primary colorectal cancer. Int J Cancer. 132(7):1679-82.

Levin B, Lieberman DA, McFarland B, Smith RA, Brooks D, Andrews KS, Dash C, Giardiello FM, Glick S, Levin TR, Pickhardt P, Rex DK, Thorson A, Winawer SJ; American Cancer Society Colorectal Cancer Advisory Group; US Multi-Society Task Force; American College of Radiology Colon Cancer Committee. 2008. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. CA Cancer J Clin. 58(3):130-60.

Ligtenberg MJ, Kuiper RP, Chan TL, Goossens M, Hebeda KM, Voorendt M, Lee TY, Bodmer D, Hoenselaar E, Hendriks-Cornelissen SJ, Tsui WY, Kong CK, Brunner HG, van Kessel AG, Yuen ST, van Krieken JH, Leung SY, Hoogerbrugge N. 2009.

REFERENCES

Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3' exons of TACSTD1. Nat Genet. 41(1):112-7.

Lin KM, Shashidharan M, Thorson AG, Ternent CA, Blatchford GJ, Christensen MA,Watson P, Lemon SJ, Franklin B, Karr B, Lynch J, Lynch HT. 1998. Cumulative incidence of colorectal and extracolonic cancers in MLH1 and MSH2 mutation carriers of hereditary nonpolyposis colorectal cancer. J Gastrointest Surg. 2(1):67-71.

Lothe RA, Peltomäki P, Meling GI, Aaltonen LA, Nyström-Lahti M, Pylkkänen L, Heimdal K, Andersen TI, Møller P, Rognum TO, Fosså SD, Haldorsen T, Langmark F, Brøgger A, de la Chapelle A, Børresen AL. 1993. Genomic instability in colorectal cancer: relationship to clinicopathological variables and family history. Cancer Res. 53(24):5849-52.

Lüchtenborg M, Weijenberg MP, Roemen GM, de Bruïne AP, van den Brandt PA, Lentjes MH, Brink M, van Engeland M, Goldbohm RA, de Goeij AF. 2004. APC mutations in sporadic colorectal carcinomas from The Netherlands Cohort Study. Carcinogenesis. 25(7):1219-26.

Lüchtenborg M, Weijenberg MP, Wark PA, Saritas AM, Roemen GM, van Muijen GN, de Bruïne AP, van den Brandt PA, de Goeij AF. 2005. Mutations in APC, CTNNB1 and K-ras genes and expression of hMLH1 in sporadic colorectal carcinomas from the Netherlands Cohort Study. BMC Cancer. 5:160.

Lynch HT, de la Chapelle A. 2003. Hereditary colorectal cancer. N Engl J Med. 348(10):919-32.

Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch JF, Lynch PM, Cavalieri RJ, Boland CR. 1993. Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. Gastroenterology. 104(5):1535-49.

Malkhosyan S, Rampino N, Yamamoto H, Perucho M. 1996. Frameshift mutator mutations. Nature. 1382(6591):499-500.

Mangold E, Pagenstecher C, Friedl W, Fischer HP, Merkelbach-Bruse S, Ohlendorf M, Friedrichs N, Aretz S, Buettner R, Propping P, Mathiak M. 2005. Tumours from MSH2 mutation carriers show loss of MSH2 expression but many tumours from MLH1 mutation carriers exhibit weak positive MLH1 staining. J Pathol. 207(4):385-95.

Marieb & Mallatt, Jon & Wilhelm, Patricia Brady. 2008. Human anatomy, 5th edition. Pearson Benhamin Cummings, San Francisco.

Marti TM, Kunz C, Fleck O. 2002. DNA mismatch repair and mutation avoidance pathways. J Cell Physiol. 191(1):28-41.

Martínez ME, Thompson P, Messer K, Ashbeck EL, Lieberman DA, Baron JA, Ahnen DJ, Robertson DJ, Jacobs ET, Greenberg ER, Cross AJ, Atkin W. 2012. One-year risk for advanced colorectal neoplasia: U.S. versus U.K. risk-stratification guidelines. Ann Intern Med. 157(12):856-64.

Mead LJ, Jenkins MA, Young J, Royce SG, Smith L, St John DJ, Macrae F, GilesGG, Hopper JL, Southey MC. 2007. Microsatellite instability markers for identifying early-onset colorectal cancers caused by germ-line mutations in DNA mismatch repair genes. Clin Cancer Res. 13(10):2865-9.

Miyaki L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, Burgart LJ, McDonnell SK, Schaid DJ, Thibodeau SN. 2003. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res. 63(17):5209-12.

REFERENCES

Miyaki M, Iijima T, Kimura J, Yasuno M, Mori T, Hayashi Y, Koike M, Shitara N, Iwama T, Kuroki T. 1999. Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer.Cancer Res. 59(18):4506-9.

Miyaki M, Iijima T, Konishi M, Sakai K, Ishii A, Yasuno M, Hishima T, Koike M,Shitara N, Iwama T, Utsunomiya J, Kuroki T, Mori T. 1999. Higher frequency of Smad4 gene mutation in human colorectal cancer with distant metastasis. Oncogene. 18(20):3098-103.

Miyaki M, Iijima T, Shiba K, Aki T, Kita Y, Yasuno M, Mori T, Kuroki T, Iwama T. 2001. Alterations of repeated sequences in 5' upstream and coding regions in colorectal tumors from patients with hereditary nonpolyposis colorectal cancer and Turcot syndrome. Oncogene. 20(37):5215-8.

Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, Aoki T, Miki Y,Mori T, Nakamura Y. 1992. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. Hum Mol Genet. 1(4):229-33.

Modrich P. 1991. Mechanisms and biological effects of mismatch repair. Annu RevGenet. 25:229-53.

Moghbeli M, Moaven O, Dadkhah E, Farzadnia M, Roshan NM, Asadzadeh-Aghdaee H, Bahar MM, Raeisossadati R, Forghanifard MM, Bakhtiari SR, Baradaran A, Abbaszadegan MR. 2011. High frequency of microsatellite instability in sporadic colorectal cancer patients in Iran. Genet Mol Res. 10(4):3520-9.

Moisio AL, Sistonen P, Weissenbach J, de la Chapelle A, Peltomäki P. 1996. Age and origin of two common MLH1 mutations predisposing to hereditary colon cancer. Am J Hum Genet. 59(6):1243-51.

Moussa SA, Moussa A, Kourda N, Mezlini A, Abdelli N, Zerimech F, Najjar T, Jilani SB, Porchet N, Ayed FB, Manai M, Buisine MP. 2011. Lynch syndrome in Tunisia:first description of clinical features and germline mutations. Int J Colorectal Dis. 26(4):455-67.

Mueller-Koch Y, Vogelsang H, Kopp R, Lohse P, Keller G, Aust D, Muders M, Gross M, Daum J, Schiemann U, Grabowski M, Scholz M, Kerker B, Becker I, Henke G, Holinski-Feder E. 2005. Hereditary non-polyposis colorectal cancer: clinical and molecular evidence for a new entity of hereditary colorectal cancer. Gut. 54(12):1733-40.

Myeroff LL, Parsons R, Kim SJ, Hedrick L, Cho KR, Orth K, Mathis M, Kinzler KW, Lutterbaugh J, Park K, Bang Y, Lee HY, Park JG, Lynch HT, Roberts AB, Vogeistein B, Markowitz SD. 1995. A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. Cancer Res. 55(23):5545-7.

Naseem H, Boylan J, Speake D, Leask K, Shenton A, Lalloo F, Hill J, Trump D, Evans DG. 2006. Inherited association of breast and colorectal cancer: limited role of CHEK2 compared with high-penetrance genes. Clin Genet. 70(5):388-95.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Colorectal cancer screening. Version 2.2013.

National Comprehensive Cancer Network (NCCN). NCCN Clinical Practice Guidelines in Oncology. Colon cancer Version 3.2014.

Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K,Utsunomiya J, Baba S, Hedge P. 1991. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science. 253(5020):665-9.

REFERENCES

Nyström-Lahti M, Kristo P, Nicolaides NC, Chang SY, Aaltonen LA, Moisio AL, Järvinen HJ, Mecklin JP, Kinzler KW, Vogelstein B, De La Chapelle A, Peltomäki P. 1995. Founding mutations and Alu-mediated recombination in hereditary colon cancer. Nat Med. 1(11):1203-6.

Ogino S, Kawasaki T, Ogawa A, Kirkner GJ, Loda M, Fuchs CS. 2007. TGFBR2 mutation is correlated with CpG island methylator phenotype in microsatellite instability-high colorectal cancer. Hum Pathol. 38(4):614-20.

Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS. 2009. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut. 58(1):90-6.

Ohue M, Tomita N, Monden T, Fujita M, Fukunaga M, Takami K, Yana I, Ohnishi T, Enomoto T, Inoue M, Slamano T, Mori T. 1994. A frequent alteration of p53 gene in carcinoma in adenoma of colon. Cancer Res. 54(17):4798-804.

Olschwang S, Hamelin R, Laurent-Puig P, Thuille B, De Rycke Y, Li YJ, Muzeau F, Girodet J, Salmon RJ, Thomas G. 1997. Alternative genetic pathways in colorectal carcinogenesis. Proc Natl Acad Sci U S A. 94(22):12122-7.

Orimo H, Nakajima E, Ikejima M, Emi M, Shimada T. 1999. Frameshift mutations and a length polymorphism in the hMSH3 gene and the spectrum of microsatellite instability in sporadic colon cancer. Jpn J Cancer Res. 90(12):1310-5.

Paskett ED, Reeves KW, Rohan TE, Allison MA, Williams CD, Messina CR, Whitlock E, Sato A, Hunt JR. 2007. Association between cigarette smoking and colorectal cancer in the Women's Health Initiative. J Natl Cancer Inst. 99(22):1729-35.

Patil DT, Bronner MP, Portier BP, Fraser CR, Plesec TP, Liu X. 2012. A five-marker panel in a multiplex PCR accurately detects microsatellite instability-high colorectal tumors without control DNA. Diagn Mol Pathol. 21(3):127-33.

Peel DJ, Ziogas A, Fox EA, Gildea M, Laham B, Clements E, Kolodner RD, Anton-Culver H. 2000. Characterization of hereditary nonpolyposis colorectal cancer families from a population-based series of cases. J Natl Cancer Inst. 92(18):1517-22.

Peixoto A, Santos C, Rocha P, Pinheiro M, Príncipe S, Pereira D, Rodrigues H, Castro F, Abreu J, Gusmão L, Amorim A, Teixeira MR. 2009. The c.156_157insAlu BRCA2 rearrangement accounts for more than one-fourth of deleterious BRCA mutations in northern/central Portugal. Breast Cancer Res Treat. 114(1):31-8.

Peltomäki P. 2001 Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Genet. 10(7):735-40.

Peltomäki P. 2001. DNA mismatch repair and cancer. Mutat Res. 488(1):77-85.

Peltomäki P. 2005. Lynch syndrome genes. Fam Cancer. 4(3):227-32.

Percesepe A, Benatti P, Roncucci L, Sassatelli R, Fante R, Ganazzi D, Bellacosa A, Genuardi M, Neri G, Viel A, Ponz de Leon M. 1997. Survival analysis in families affected by hereditary non-polyposis colorectal cancer. Int J Cancer. 71(3):373-6.

Perucho M. 1996. Cancer of the microsatellite mutator phenotype. Biol Chem. 377(11):675-84.

Perucho M. 1996. Microsatellite instability: the mutator that mutates the other mutator. Nat Med. 2(6):630-1.

REFERENCES

Peterlongo P, Nafa K, Lerman GS, Glogowski E, Shia J, Ye TZ, Markowitz AJ, Guillem JG, Kolachana P, Boyd JA, Offit K, Ellis NA. 2003. MSH6 germline mutations are rare in colorectal cancer families. Int J Cancer. 107(4):571-9.

Petras RE. Frankel WI. 2009. Large Intestine (Colon). In Weidner N. Cote RJ. Suster S. Weiss LM. Modern Surgical Pathology, 2nd edition. Elsevier Health Sciences.

Pinheiro M, Ahlquist T, Danielsen SA, Lind GE, Veiga I, Pinto C, Costa V, Afonso L, Sousa O, Fragoso M, Santos L, Henrique R, Lopes P, Lopes C, Lothe RA, Teixeira MR. 2010. Colorectal carcinomas with microsatellite instability display a different pattern of target gene mutations according to large bowel site of origin. BMC Cancer. 10:587.

Planck M, Wenngren E, Borg A, Olsson H, Nilbert M. 2000. Somatic frameshift alterations in mononucleotide repeat-containing genes in different tumor types from an HNPCC family with germline MSH2 mutation. Genes Chromosomes Cancer. 29(1):33-9.

Potocnik U, Glavac D, Ravnik-Glavac M. 2003. Identification of novel genes with somatic frameshift mutations within coding mononucleotide repeats in colorectal tumors with high microsatellite instability. Genes Chromosomes Cancer. 36(1):48-56.

Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. 1992. APC mutations occur early during colorectal tumorigenesis. Nature. 359(6392):235-7.

Roth S, Johansson M, Loukola A, Peltomäki P, Järvinen H, Mecklin JP, Aaltonen LA. 2000. Mutation analysis of SMAD2, SMAD3, and SMAD4 genes in hereditary non-polyposis colorectal. J Med Genet. 37(4):298-300.

Rowan AJ, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, Bicknell D, Bodmer WF, Tomlinson IP. 2000. APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". Proc Natl Acad Sci U S A. 97(7):3352-7.

Ruckert S, Hiendlmeyer E, Brueckl WM, Oswald U, Beyser K, Dietmaier W, HaynlA, Koch C, Rüschoff J, Brabletz T, Kirchner T, Jung A. 2002. T-cell factor-4 frameshift mutations occur frequently in human microsatellite instability-high colorectal carcinomas but do not contribute to carcinogenesis. Cancer Res. 62(11):3009-13.

Saito-Diaz K, Chen TW, Wang X, Thorne CA, Wallace HA, Page-McCaw A, Lee E. 2013. The way Wnt works: components and mechanism. Growth Factors. 31(1):1-31.
Salahshor S, Kressner U, Påhlman L, Glimelius B, Lindmark G, Lindblom A. 1999. Colorectal cancer with and without microsatellite instability involves different genes. Genes Chromosomes Cancer. 26(3):247-52.

Samadder NJ, Vierkant RA, Tillmans LS, Wang AH, Weisenberger DJ, Laird PW, Lynch CF, Anderson KE, French AJ, Haile RW, Potter JD, Slager SL, Smyrk TC, Thibodeau SN, Cerhan JR, Limburg PJ. 2013. Associations between colorectal cancer molecular markers and pathways with clinicopathologic features in older women. Gastroenterology. 145(2):348-56.

Samowitz WS, Curtin K, Lin HH, Robertson MA, Schaffer D, Nichols M, Gruenthal K, Leppert MF, Slattery ML. 2001. The colon cancer burden of genetically defined hereditary nonpolyposis colon cancer. Gastroenterology. 121(4):830-8.

Samowitz WS, Curtin K, Ma KN, Schaffer D, Coleman LW, Leppert M, Slattery ML. 2001. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. Cancer Epidemiol Biomarkers Prev. 10(9):917-23.

REFERENCES

Samowitz WS, Curtin K, Neuhausen S, Schaffer D, Slattery ML. 2002. Prognostic implications of BAX and TGFBRII mutations in colon cancers with microsatellite instability. Genes Chromosomes Cancer. 35(4):368-71.

Schwartz S Jr, Yamamoto H, Navarro M, Maestro M, Reventós J, Perucho M. 1999. Frameshift mutations at mononucleotide repeats in caspase-5 and other target genes in endometrial and gastrointestinal cancer of the microsatellite mutator phenotype. Cancer Res. 59(12):2995-3002.

Senba S, Konishi F, Okamoto T, Kashiwagi H, Kanazawa K, Miyaki M, Konishi M, Tsukamoto T. 1998. Clinicopathologic and genetic features of nonfamilial colorectal carcinomas with DNA replication errors. Cancer. 82(2):279-85.

Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. 2007. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A. 104(47):18654-9.

Shia J, Ellis NA, Paty PB, Nash GM, Qin J, Offit K, Zhang XM, Markowitz AJ, Nafa K, Guillem JG, Wong WD, Gerald WL, Klimstra DS. 2003. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. Am J Surg Pathol. 27(11):1407-17.

Shima K, Morikawa T, Yamauchi M, Kuchiba A, Imamura Y, Liao X, Meyerhardt JA, Fuchs CS, Ogino S. 2011. TGFBR2 and BAX mononucleotide tract mutations, microsatellite instability, and prognosis in 1072 colorectal cancers. PLoS One. 6(9):e25062.

Shin KH, Park YJ, Park JG. 2000. Mutational analysis of the transforming growth factor beta receptor type II gene in hereditary nonpolyposis colorectal cancer and early-onset colorectal cancer patients. Clin Cancer Res. 6(2):536-40.

Sinicrope FA, Sargent DJ. 2012. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin Cancer Res. 18(6):1506-12.

Smith G, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. 2002. Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci U S A. 99(14):9433-8.

Smyrk TC, Watson P, Kaul K, Lynch HT. 2001. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer. 91(12):2417-22.

Sobin LH, Gospodarowicz MK, Wittekind C. 2009. The TNM Classification of Malignant Tumours, 7th edition. UICC.

Sparks AB, Morin PJ, Vogelstein B, Kinzler KW. 1998. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. Cancer Res. 58(6):1130-4.

Sun S, Greenwood CM, Thiffault I, Hamel N, Chong G, Foulkes WD. 2005. The HNPCC associated MSH2*1906G-->C founder mutation probably originated between 1440 CE and 1715 CE in the Ashkenazi Jewish population. J Med Genet. 42(10):766-8.

Syed Sameer A, Shah ZA, Abdullah S, Chowdri NA, Siddiqi MA. 2011. Analysis of molecular aberrations of Wnt pathway gladiators in colorectal cancer in the Kashmiri population. Hum Genomics. 5(5):441-52.

Taylor DP, Burt RW, Williams MS, Haug PJ, Cannon-Albright LA. 2010. Population-based family history-specific risks for colorectal cancer: a constellation approach. Gastroenterology. 138(3):877-85.

REFERENCES

Thibodeau SN, Bren G, Schaid D. 1993. Microsatellite instability in cancer of the proximal colon. Science. 1993 260(5109):816-9.

Thompson BA, Spurdle AB, Plazzer JP, Greenblatt MS, Akagi K, Al-Mulla F, Bapat B, Bernstein I, Capellá G, den Dunnen JT, du Sart D, Fabre A, Farrell MP, Farrington SM, Frayling IM, Frebourg T, Goldgar DE, Heinen CD, Holinski-Feder E, Kohonen-Corish M, Robinson KL, Leung SY, Martins A, Moller P, Morak M, Nystrom M, Peltomaki P, Pineda M, Qi M, Ramesar R, Rasmussen LJ, Royer-Pokora B, Scott RJ, Sijmons R, Tavtigian SV, Tops CM, Weber T, Wijnen J, Woods MO, Macrae F, Genuardi M; InSiGHT. 2014. Application of a 5-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants in the InSiGHT locus-specific database. Nat Genet. 46(2):107-15.

Thorstensen L, Lind GE, Løvig T, Diep CB, Meling GI, Rognum TO, Lothe RA. 2005. Genetic and epigenetic changes of components affecting the WNT pathway in colorectal carcinomas stratified by microsatellite instability. Neoplasia. 7(2):99-108.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. 1999. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A. 96(15):8681-6.

Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Rüschoff J, Fishel R, Lindor NM, Burgart LJ, Hamelin R, Hamilton SR, Hiatt RA, Jass J, Lindblom A, Lynch HT, Peltomaki P, Ramsey SD, Rodriguez-Bigas MA, Vasen HF, Hawk ET, Barrett JC, Freedman AN, Srivastava S. 2004. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 96(4):261-8.

Umar A, Koi M, Risinger JI, Glaab WE, Tindall KR, Kolodner RD, Boland CR, Barrett JC, Kunkel TA. 1997. Correction of hypermutability,N-methyl-N'-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing

chromosome 2 into human tumor cells with mutations in MSH2 and MSH6. Cancer Res. 57(18):3949-55.

Vaccaro CA, Bonadeo F, Roverano AV, Peltomaki P, Bala S, Renkonen E, Redal MA, Mocetti E, Mullen E, Ojea-Quintana G, Benati ML, Rivello HG, Clark MB, Lynch JF, Lynch HT. 2007. Hereditary nonpolyposis colorectal cancer (Lynch Syndrome) in Argentina: report from a referral hospital register. Dis Colon Rectum. 50(10):1604-11.

van der Luijt RB, van Zon PH, Jansen RP, van der Sijs-Bos CJ, Wárlám-Rodenhuis CC, Ausems MG. 2001. De novo recurrent germline mutation of the BRCA2 gene in a patient with early onset breast cancer. J Med Genet. 38(2):102-5.

van Duijnhoven FJ, Bueno-De-Mesquita HB, Ferrari P, Jenab M, Boshuizen HC, Ros MM, Casagrande C, Tjønneland A, Olsen A, Overvad K, Thorlacius-Ussing O, Clavel-Chapelon F, Boutron-Ruault MC, Morois S, Kaaks R, Linseisen J, Boeing H, Nöthlings U, Trichopoulou A, Trichopoulos D, Misirli G, Palli D, Sieri S, Panico S, Tumino R, Vineis P, Peeters PH, van Gils CH, Ocké MC, Lund E, Engeset D, Skeie G, Suárez LR, González CA, Sánchez MJ, Dorronsoro M, Navarro C, Barricarte A, Berglund G, Manjer J, Hallmans G, Palmqvist R, Bingham SA, Khaw KT, Key TJ, Allen NE, Boffetta P, Slimani N, Rinaldi S, Gallo V, Norat T, Riboli E. 2009. Fruit, vegetables, and colorectal cancer risk: the European Prospective Investigation into Cancer and Nutrition. Am J Clin Nutr. 89(5):1441-52.

Vasen HF, Möslein G, Alonso A, Bernstein I, Bertario L, Blanco I, Burn J, Capella G, Engel C, Frayling I, Friedl W, Hes FJ, Hodgson S, Mecklin JP, Møller P, Nagengast F, Parc Y, Renkonen-Sinisalo L, Sampson JR, Stormorken A, Wijnen J. 2007. Guidelines for the clinical management of Lynch syndrome (hereditarynon-polyposis cancer). J Med Genet. 44(6):353-62.

REFERENCES

Vasen HF, Watson P, Mecklin JP, Lynch HT. 1999. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 116(6):1453-6.

Vasovcak P, Pavlikova K, Sedlacek Z, Skapa P, Kouda M, Hoch J, Krepelova 2011. A .Molecular genetic analysis of 103 sporadic colorectal tumours in Czech patients. PLoS One. 6(8):e24114.

Wagner A, Barrows A, Wijnen JT, van der Klift H, Franken PF, Verkuijlen P, Nakagawa H, Geugien M, Jaghmohan-Changur S, Breukel C, Meijers-Heijboer H, Morreau H, van Puijenbroek M, Burn J, Coronel S, Kinarski Y, Okimoto R, Watson P, Lynch JF, de la Chapelle A, Lynch HT, Fodde R. 2003. Molecular analysis of hereditary nonpolyposis colorectal cancer in the United States: high mutation detection rate among clinically selected families and characterization of an American founder genomic deletion of the MSH2 gene. Am J Hum Genet. 72(5):1088-100.

Wagner A, Hendriks Y, Meijers-Heijboer EJ, de Leeuw WJ, Morreau H, Hofstra R, Tops C, Bik E, Bröcker-Vriends AH, van Der Meer C, Lindhout D, Vasen HF, Breuning MH, Cornelisse CJ, van Krimpen C, Niermeijer MF, Zwinderman AH, Wijnen J, Fodde R. Atypical 2001. HNPCC owing to MSH6 germline mutations: analysis of a large Dutch pedigree. J Med Genet. 38(5):318-22.

Wang Y, Friedl W, Lamberti C, Jungck M, Mathiak M, Pagenstecher C, Propping P, Mangold E. 2003. Hereditary nonpolyposis colorectal cancer: frequent occurrence of large genomic deletions in MSH2 and MLH1 genes. Int J Cancer. 103(5):636-41.

Wei EK, Giovannucci E, Wu K, Rosner B, Fuchs CS, Willett WC, Colditz GA. 2004. Comparison of risk factors for colon and rectal cancer. Int J Cancer. 108(3):433-42.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett

B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 38(7):787-93.

Wheeler JM, Loukola A, Aaltonen LA, Mortensen NJ, Bodmer WF. 2000. The role of hypermethylation of the hMLH1 promoter region in HNPCC versus MSI+ sporadic colorectal cancers. J Med Genet. 37(8):588-92.

Whitehall VL, Wynter CV, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, Jass JR. 2002. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. Cancer Res. 62(21):6011-4.

Woerner SM, Gebert J, Yuan YP, Sutter C, Ridder R, Bork P, von Knebel Doeberitz M. 2001. Systematic identification of genes with coding microsatellites mutated in DNA mismatch repair-deficient cancer cells. Int J Cancer. 93(1):12-9.

Woodford-Richens KL, Rowan AJ, Gorman P, Halford S, Bicknell DC, Wasan HS, Roylance RR, Bodmer WF, Tomlinson IP. 2001. SMAD4 mutations in colorectal cancer probably occur before chromosomal instability, but after divergence of the microsatellite instability pathway. Proc Natl Acad Sci U S A. 98(17):9719-23.

Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, van Der Zee AG, Hollema H, Kleibeuker JH, Buys CH, Hofstra RM. 1999. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. Am J Hum Genet. 65(5):1291-8.

Yagi OK, Akiyama Y, Nomizu T, Iwama T, Endo M, Yuasa Y. 1998. Proapoptotic gene BAX is frequently mutated in hereditary nonpolyposis colorectal cancers but not in adenomas. Gastroenterology. 114(2):268-74.

REFERENCES

Yamamoto H, Sawai H, Weber TK, Rodriguez-Bigas MA, Perucho M. 1998. Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. Cancer Res. 58(5):997-1003.

Yashima K, Nakamori S, Murakami Y, Yamaguchi A, Hayashi K, Ishikawa O, Konishi Y, Sekiya T. 1994. Mutations of the adenomatous polyposis coli gene in the mutation cluster region: comparison of human pancreatic and colorectal cancers. Int JCancer. 59(1):43-7.

Yearsley M, Hampel H, Lehman A, Nakagawa H, de la Chapelle A, Frankel WL. 2006. Histologic features distinguish microsatellite-high from microsatellite-low and microsatellite-stable colorectal carcinomas, but do not differentiate germline mutations from methylation of the MLH1 promoter. Hum Pathol. 37(7):831-8.

Young J, Simms LA, Biden KG, Wynter C, Whitehall V, Karamatic R, George J, Zawel L, Dai JL, Buckhaults P, Zhou S, Kinzler KW, Vogelstein B, Kern SE. 1998. Human Smad3 and Smad4 are sequence-specific transcription activators. Mol Cell. 1(4):611-7.

Zhou S, Buckhaults P, Zawel L, Bunz F, Riggins G, Dai JL, Kern SE, Kinzler KW, Vogelstein B. 1998. Targeted deletion of Smad4 shows it is required for transforming growth factor beta and activin signaling in colorectal cancer cells. Proc NatlAcad Sci U S A. 95(5):2412-6.

Zhou XP, Kuismanen S, Nystrom-Lahti M, Peltomaki P, Eng C. 2002. Distinct PTEN mutational spectra in hereditary non-polyposis colon cancer syndrome-related endometrial carcinomas compared to sporadic microsatellite unstable tumors. Hum Mol Genet. 11(4):445-50.